VOLUME 28 NUMBER 1

PLANT PHYSIOLOGY

JANUARY, 1953

CONTENTS

Influence of Visible and Near Infrared Radiant Energy on Organ Development and Pigment Synthesis in Bean and Corn. R. B. Withrow, W. H. Klein, L. Price and V. Elstad, Smithsonian Institution, Washington, D. C. 1

Studies on the Chemistry of the Living Bark of the Black Locust in Relation to Its Frost Hardiness. III. The Validity of Plasmolysis and Desiccation Tests for Determining the Frost Hardiness of Bark Tissue. D. Siminovitch and D. R. Briggs, Division of Agricultural Biochemistry, University of Minnesota, St. Paul, Minnesota 15

The Sprouting of Sweetgum in Relation to Season of Cutting and Carbohydrate Content. Karl F. Wenger, Southeastern Forest Experiment Station, Franklin, Virginia 35

The Physiological Action of Smog on Plants. I. Initial Growth and Transpiration Studies. Helen Garman Koritz and F. W. Went, Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California 50

Some Respiratory Characteristics of the Blue-Green Alga, Anabaena. George C. Webster and Albert W. Frenkel, Department of Botany, University of Minnesota, Minneapolis, Minnesota 63

The Metabolism of Foliar-Applied Urea. I. Relative Rates of $C^{14}O_2$ Production by Certain Vegetable Plants Treated with Labeled Urea. O. N. Hinsvark, S. H. Wittwer and H. B. Tukey, Department of Horticulture, Michigan State College, East Lansing, Michigan 70

(*Continued on Cover Page Four*)

PUBLISHED QUARTERLY

BY

THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

AT 111 E. CHESTNUT ST., LANCASTER, PA.

Entered as second-class matter March 25, 1926, at the post office at Lancaster, Pa., under the Act of August 24, 1912.

Accepted for mailing at the special rate of postage provided for in paragraph 4, section 538, Authorized April 9, 1926.

PLANT PHYSIOLOGY

A QUARTERLY JOURNAL
DEVOTED TO ALL PHASES OF PLANT PHYSIOLOGY

Established 1926

WALTER F. LOEHWING, *Editor-in-chief*, 1954
State University of Iowa

ASSOCIATE EDITORS

C. E. WADLEIGH, 1955
U.S.D.A. Plant Industry Station
Beltsville, Maryland

M. W. PARKER, 1954
U.S.D.A. Plant Industry Station
Beltsville, Maryland

J. B. BIALE, 1955
University of California
Los Angeles, California

H. B. VICKERY, 1956
Connecticut Agricultural
Experiment Station

F. P. CULLINAN, 1955
U.S.D.A. Plant Industry Station
Beltsville, Maryland

C. S. FRENCH, 1953
Carnegie Institution
Stanford, California

ROBERT M. MUIR, *Secretary of the Editorial Board*, 1956
State University of Iowa

J. BONNER, 1953
California Institute of Technology
Pasadena, California

E. A. BORTHWICK, 1954
U.S.D.A. Plant Industry Station
Beltsville, Maryland

P. J. KRAMER, 1953
Duke University
Durham, North Carolina

W. E. LOOMIS, 1955
Iowa State College
Ames, Iowa

K. C. HAMNER, 1954
University of California
Los Angeles, California

R. EMERSON, 1953
University of Illinois
Urbana, Illinois

The pages of this journal are open to papers of merit dealing with any phase of Plant Physiology. Membership in the Society is not required of contributors, but non-members are required to pay a fee of $20.00 for each paper printed. Manuscripts must be typewritten, and must have the approval of the Editorial Board. Papers must conform to the limitations established by the Society at the St. Louis meeting, 1935. Proofs should be returned with necessary corrections, and reprints ordered when the proof is returned. Reprints can be furnished with or without covers at cost of production. Correspondence with reference to editorial matters should be addressed to the Editor, Dr. WALTER F. LOEHWING, Department of Botany, State University of Iowa, Iowa City, Iowa.

Subscription price, $10.00 a year. Single copies, $3.00 post free. Members of the American Society of Plant Physiologists receive the journal in connection with dues paid to the Society. Missing numbers will be supplied if lost in the mail, provided that written notice is received within one month of the date of issue; otherwise they will be furnished at the single copy rate, as long as they are available.

Correspondence concerning membership and its privileges, dues, change-of-address, etc., should be addressed to the Executive Secretary-Treasurer, Dr. J. FISHER STANFIELD, Miami University, Oxford, Ohio. Business correspondence regarding subscriptions, back numbers, change-of-address of subscribers, etc., may be addressed to PLANT PHYSIOLOGY, 111 N. Chestnut St., Lancaster, Pa., or to the Executive Secretary-Treasurer at the address above. All matters pertaining to nominations, elections, programs, and meetings of the society should be addressed to the Secretary, Dr. A. S. CRAFTS, University of California, Davis, California.

PLANT PHYSIOLOGY

VOLUME 28 JANUARY, 1953 NUMBER 1

INFLUENCE OF VISIBLE AND NEAR INFRARED RADIANT ENERGY ON ORGAN DEVELOPMENT AND PIGMENT SYNTHESIS IN BEAN AND CORN[1]

R. B. WITHROW, W. H. KLEIN, L. PRICE AND V. ELSTAD

SMITHSONIAN INSTITUTION, WASHINGTON, D. C.

Received May 19, 1952

The principal morphogenic responses of dicotyledonous seedlings to red radiant energy have been reported to be an inhibition in growth of hypocotyl when present, an accelerated straightening of the plumular hook, an accelerated leaf expansion, an increased rate of epicotyl development, and a generally increased rate of tissue maturation (**7, 10, 13, 15**). In the monocotyledons, the most conspicuous response is a reduced growth rate of the first internode (**11**). The longer wave lengths of the visible spectrum also cause chlorophyll synthesis and photosynthesis. Due principally to the overlapping of the action spectra of these three photochemical reactions, it has been impossible to separate photomorphogenesis from the other two reactions. Consequently, when red radiant energy is used, the biochemical investigation of photomorphogenesis is complicated by the products of three photochemical reactions occurring simultaneously. It, therefore, would be advantageous to be able to separate photomorphogenesis from chlorophyll synthesis and photosynthesis. Attempts to accomplish this separation by the use of albino or chlorophyll-deficient plants have not been successful since all plants so far tested in this laboratory have contained significant quantities of chlorophyll.

The curve for chlorophyll synthesis in barley seedlings as reported by KOSKI *et al.* (**9**) shows a long wave length limit of ca. 680 mμ. Action spectra curves for photosynthesis as determined by EMERSON and LEWIS (**4**) for Chlorella, and CHEN (**2**) for the Hill reaction in Swiss chard chloroplasts indicate limits of 700 to 710 mμ. On the other hand, PARKER *et al.* (**10**) found evidence of a limit of 720 to 730 mμ for leaf expansion in pea seedlings, and data on the inhibition of the first internode of Avena by GOODWIN and OWENS (**7**), and WEINTRAUB and PRICE (**13**) likewise indicate that the longer wave length limit for the growth reactions of the seedling occurs somewhat further toward the infrared than the limits for either chlorophyll synthesis or photosynthesis. Using a long wave pass dyed gelatin filter

[1] Published by permission of the Secretary of the Smithsonian Institution.

which began to transmit at ca. 720 mμ, WITHROW (**15**) reported that bean seedlings expanded their leaves, lost the plumular hooks, and developed shortened hypocotyls without any visible green coloration. Similar results were obtained with other seedlings and with shoots of potato tubers. However, bean leaves which had been exposed to the far red contained appreciable quantities of chlorophyll as shown by subsequent acetone extraction. These observations indicated that it might be possible to separate photomorphogenesis from chlorophyll synthesis and photosynthesis by the use of more refined filter techniques.

In addition to inducing various growth responses in young shoots, converting protochlorophyll to chlorophyll, and supporting photosynthesis, visible radiant energy has been shown to cause an increased rate of synthesis of such diverse pigments as the carotenoids and the anthocyanins. These pigments usually are synthesized in the dark, but irradiation greatly increases the rate of synthesis in some tissues (**1, 5, 12**) and the evidence points to photochemically accelerated synthesis of both groups of pigments by other than photosynthetic products, although the question is by no means definitely resolved.

The present report represents the results of experiments designed to achieve photochemical separation of the photomorphogenic reactions from chlorophyll synthesis and photosynthesis. In the course of examining acetone and aqueous extracts of the plant material, it was observed that the spectral regions which were most effective in inducing the growth reactions also altered the pigment picture as regards the chloroplast pigments and anthocyanin. Data on these pigment changes are presented, although no direct relationship to photomorphogenesis is as yet clear.

Materials and methods

The plant material was grown in subirrigation nutrient culture (**18**) in eight growth chambers placed in a room where the temperature and humidity were controlled. These chambers were constructed of marine plywood as shown in figure 1 and were enameled white inside and black outside. A small blower in the space below the culture pan of each compartment forced air from the room into the compartment and out through baffles near the top of the chamber. Warm air from the irradiation system in the top of the compartment was discharged into the room before reaching the plants. A small amount of filtered fresh air from outside the building was forced into the room continuously.

The complete irradiation system for each compartment consisted of internal reflector lamps mounted in a water-cooled glass-bottomed tank, a 10-cm. aqueous primary filter, and a dyed gelatin secondary filter. The irradiation system used is described elsewhere (**16**). The dyed gelatin secondary filters were prepared in this laboratory by a technique also presented in detail elsewhere (**17**). The composition of the filters used and their per cent. transmittances are given in table I and figure 2, respectively. These filters are stable and have withstood months of intense irradiation with little

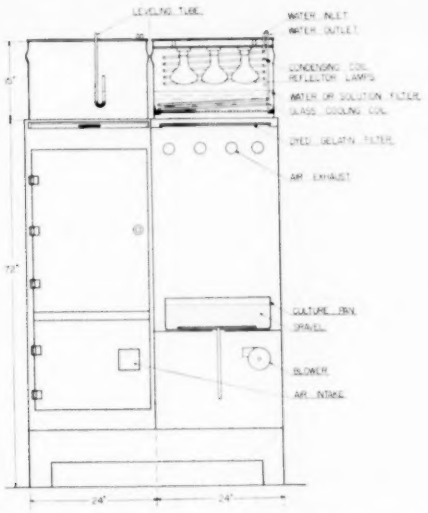

FIG. 1. Details of two growth chambers and two water-cooled lamp systems. On the left is an external view of a growth chamber and lamp tank; on the right are constructional details of a chamber and lamp system.

measurable change. The wave length designation for each far red filter was arbitrarily taken as that point, to the nearest 5 mμ, where the transmittance was ca. 0.5%.

All irradiances were measured with a fast spectrometer (Perkin-Elmer) type thermocouple operating into a contact modulation amplifier (Liston-Becker) and a one milliampere strip chart recorder for direct deflection (Esterline-Angus). The thermocouple was mounted in a thermally insu-

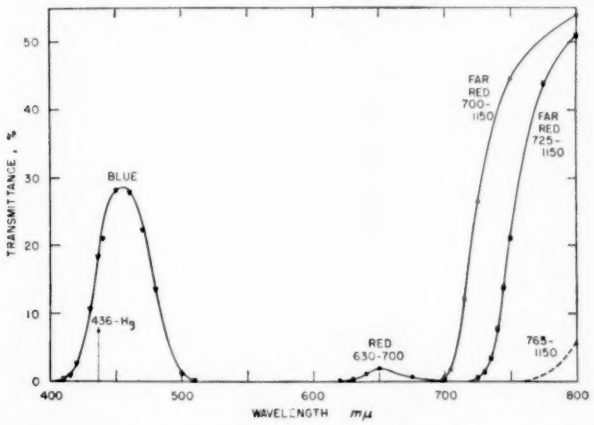

FIG. 2. Transmission curves of the radiation filters. Ordinate is per cent. transmittance (balanced against air) of the complete system of primary and secondary filters.

TABLE I

PROPERTIES OF THE IRRADIATION SYSTEMS.

Wave length limits	Irradiance*	Radiation source	Primary filter aqueous	Secondary filter dyed gelatin
Far red 765–1150 mμ	300 μw./cm.²	3 × 150-watt reflector flood, incandescent	10 cm. water	Pontamine diazo blue, 1.0 mg./cm.² Pontamine fast red 8 BL, 0.5 mg./cm.²
Far red 725–1150 mμ	150 μw./cm.²	150-watt reflector flood, incandescent	10 cm. water	Acid green no. 1854, 0.8 mg./cm.² Pontamine fast red 8 BL, 0.7 mg./cm.²
Far red 725–1150 mμ	450 μw./cm.²	300-watt reflector flood, incandescent	10 cm. water	Acid green no. 1854, 0.8 mg./cm.² Pontamine fast red 8 BL, 0.7 mg./cm.²
Far red 725–1150 mμ	1500 μw./cm.²	3 × 300-watt reflector flood, incandescent	10 cm. water	Acid green no. 1854, 0.8 mg./cm.² Pontamine fast red 8 BL, 0.7 mg./cm.²
Far red 700–1150 mμ	450 μw./cm.²	300-watt reflector flood, incandescent	10 cm. water	Pontacyl brilliant blue E, 1.5 mg./cm.² Pontamine fast red 8 BL, 0.5 mg./cm.²
Red 630–700 mμ	2 μw./cm.²	150-watt reflector flood, incandescent	10 cm. 1% $CuSO_4 \cdot 5 H_2O$	Pontamine fast red 8 BL, 2.0 mg./cm.²
Red 630–700 mμ	0.05 μw./cm.²	150-watt reflector flood, incandescent	10 cm. 1% $CuSO_4 \cdot 5 H_2O$	Pontamine fast red 8 BL, 2.0 mg./cm.²
Blue 436 mμ	2 μw./cm.²	100-watt reflector flood, mercury arc	10 cm. 5% $CuSO_4 \cdot 5 H_2O$	Victoria pure blue BO, 0.2 mg./cm.² 8-Hydroxyquinoline sulphate, 1.5 mg./cm.²
Blue 436 mμ	0.05 μw./cm.²	100-watt reflector flood, mercury arc	10 cm. 5% $CuSO_4 \cdot 5 H_2O$	Victoria pure blue BO, 0.2 mg./cm.² 8-Hydroxyquinoline sulphate, 1.5 mg./cm.²

*Neutral filters were employed to obtain the indicated values.

lated wood case with a metal shutter. A water cell 3 cm. thick was placed between the receiver and shutter to remove energy of long wave lengths from warm filters and other objects at slight thermal disequilibrium with the thermocouple. The thermocouple was calibrated, without the water cell, against a standard lamp from the National Bureau of Standards, with a factor applied to correct for losses by the cell. For the visible spectrum, this factor amounted to about 10%.

Since the far red filters transmitted all wave lengths from the wave length designation of the filter to 1150 mμ and the nonselective thermocouple measured total transmitted energy, no indication of the level of the effective far red energy is given by such thermocouple measurements. Therefore, in order to avoid uncertainty as to the relative irradiance at the short wave length limit of the filters for the far red series (725 mμ), a photocell detector (Welch Densichron) with an S-1 photosurface sensitive to the near infrared was used. The photocell was covered with an interference filter two inches by two inches (Bausch and Lomb) with a peak transmission at 720 mμ, limiting the detector sensitivity to a narrow region from ca. 710 to 730 mμ. The energy values in this region then were adjusted until the desired ratios were obtained; in most cases, the total irradiance values were in the same ratios. Serious deviations occurred only when there was a difference in lamp color temperature sufficient to produce a difference in spectral energy distribution of the sources.

Bean (*Phaseolus vulgaris*), variety Black Valentine, and a hybrid field corn (*Zea mays*) variety U.S. 13, were used in all experiments. The seedlings were grown in gravel culture beds two feet square and were subirrigated automatically every six hours. During germination the beds were subirrigated with tap water and the temperature maintained at 25° C and 70% relative humidity. When the seedlings had just emerged from the gravel, the temperature was reduced to 20° C and a complete nutrient solution (**18**) was used. For the remainder of the experimental period, irradiation was continuous with no intervening dark periods.

Seedlings of bean and corn were exposed for eight and five days, respectively, to five spectral regions: the 436 mμ line of the mercury arc; a red band between ca. 630 and 700 mμ; and three far red bands from ca. 700 mμ, ca. 725 mμ, and ca. 765 mμ. In all the far red treatments, the long wave length was limited at ca. 1150 mμ by a 10-cm. water filter. The 436 mμ line was selected for the blue treatment because the action spectra of leaf growth in pea (**10**) and first internode shortening in Avena (**7**) both show a minimum effect in the region of 450 to 500 mμ. The 436 mμ line is close enough to this region to anticipate that comparable results would be secured. The red band from ca. 630 to 700 mμ coincides with the region of maximum effectiveness for the photomorphogenic response and includes the red maximum for chlorophyll synthesis. This region at 2 μw./cm.^{2} or 0.05 μw./cm.^{2} therefore served as a reference point with which to compare the relative photomorphogenic effectiveness of the blue and far red regions.

In bean, the leaf blade alone was used for chloroplast pigment analyses, but in corn, the entire excised shoot was used. The portions to be extracted were removed from the plants in complete darkness and weighed in tight aluminum cans. They were immediately macerated in the dark in 50 ml. of acetone in a Waring Blendor for five minutes at 2° C and the slurry was filtered with suction. The acetone filtrate was added to 40 ml. of ethyl ether and washed with water three times in a 1000-ml. separatory funnel by the method used by Koski *et al.* (**9**). The ether solutions were made up to equal volumes per unit fresh weight of tissue and the absorbancy (**6**) or density ($\log I_o/I$) was determined in a Beckman spectrophotometer at selected wave lengths. For protochlorophyll and chlorophyll, absorbancies were measured at 625, 645, 665, and 690 mμ in 10-cm. cells. The following equations were used for determining the concentrations in micrograms per gram of fresh weight of tissue and were adapted from those developed by Koski *et al.* (**8**) as follows:

$$C_p = (-3.0\,A_{665} - 4.1\,A_{645} + 25\,A_{625})\,V/Wb$$

$$C_{ch} = (7.9\,A_{665} + 17\,A_{645} - 0.56\,A_{625})\,V/Wb$$

where C_p = protochlorophyll in μg./gm. fresh weight, C_{ch} = chlorophyll (a + b) in μg./gm. fresh weight, A = absorbancy or density at indicated wave length in millimicrons, V = volume of solution in milliliters, W = fresh weight of tissue in grams, b = length of absorption cell in centimeters.

The absorbancy value at 690 mμ was used as a measure of the scattering resulting from colloidally dispersed material which sometimes appeared due to incomplete filtering or separation of the ether solution into two phases. No pigments with appreciable absorption at 690 mμ are present, and an appreciable absorbancy at this wave length indicates energy loss by scattering. It therefore serves as an index of the error entering the determinations of absorbancy.

TABLE II

STEM LENGTH AND DISTRIBUTION OF DRY MATTER IN BEAN SEEDLINGS UNDER VARIOUS IRRADIATION CONDITIONS FOR 11 DAYS. VALUES ARE AVERAGES FOR 100 PLANTS

Irradiation treatment	Average stem length			Average dry weight					Dry weight
	Hypocotyl	Epicotyl	Total	Leaves	Epicotyl	Cotyledons	Hypocotyl	Total shoot	
	cm.	*cm.*	*cm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	%
Dark	25	11	36	14	31	38	85	168	5.1
Far red, 725 mμ 450 μw./cm.^{2}	18	17	35	26	40	26	69	161	5.6
Far red, 700 mμ 450 μw./cm.^{2}	13	14	27	33	33	25	65	156	6.1
Red, 630–700 mμ 2 μw./cm.^{2}	14	12	26	39	26	27	59	151	6.0
Blue, 436 mμ 2 μw./cm.^{2}	20	14	34	21	36	27	78	162	5.1

The carotene absorbancy values were determined at 440 mμ in ethyl ether, using 1-cm. cells. The quantities in micrograms per gram of fresh weight were calculated by using the value of 200 gm./l./cm. for the specific absorbancy of carotene (**19**), which was taken as an approximation for crude mixtures.

Anthocyanin was extracted separately from the hypocotyl and cotyledon portions of bean with 0.1 *N* HCl by the method of THIMANN and EDMONDSON (**12**). The extraction period was usually 48 hours at about 25° C. Bean hypocotyls were cut into sections 3 to 5 cm. long for extraction, whereas the cotyledons were extracted without sectioning. It was found that macerated tissues produced solutions too opalescent for precise photometric assay. The relative anthocyanin values were determined by measuring the absorbancy at 520 mμ for solutions containing the same fresh weight of tissue per liter. The values given in tables III and V are therefore proportional to the anthocyanin content per unit of fresh weight.

Results and discussion

GROWTH RESPONSES

The results on stem lengths and dry weight distribution in bean obtained with the various irradiation treatments are presented in table II. All irradiation treatments produced some inhibition of the length and of dry weight of the hypocotyl, an increase in fresh and dry weight of the leaves, and a decrease in weight of the cotyledons, as compared to plants grown in complete darkness. The maximum photomorphogenic response was induced by 2 μw./cm.^{2} red (630 to 700 mμ) irradiance and the minimum by the blue (436 mμ) at the same irradiance. The treatment with the 700 mμ irradiance at 450 μw./cm.^{2} was next to the red in effectiveness.

TABLE III

FRESH WEIGHT AND PIGMENT CONTENT OF BEAN SEEDLINGS UNDER VARIOUS IRRADIATION CONDITIONS FOR 11 DAYS.

Irradiation treatment	Average fresh weight of leaves	Leaf pigment per gram fresh weight				Relative concentration of anthocyanin	
		Carotene	Protochlorophyll (P)	Chlorophyll (C)	P/C	Hypocotyl	Cotyledons
	mg.	*μg.*	*μg.*	*μg.*			
Dark	90	120	12	0.0	.	5	12,000
Far red, 725 mμ 450 μw./cm.^{2}	130	180	16	1.2	13	65	17,000
Far red, 700 mμ 450 μw./cm.^{2}	240	130	3	4.8	0.6	120	17,000
Red, 630–700 mμ 2 μw./cm.^{2}	270	....	....	420	..	90	15,000
Blue, 436 mμ 2 μw./cm.^{2}	115	....	....	260	....	45	14,000

The morphological responses to varying irradiances of far red (725 mμ) are graphed in figure 3. Cotyledon weight decreased and length and weight of the epicotyl and weight of the leaves increased with irradiance, although nearly the maximum response occurred at 150 or 450 μw./cm.^{2} This result indicates saturation for this wave band at these irradiances, excepting for the hypocotyl, which decreased progressively in length and weight with irradiance to the highest value used. The fresh weights of the bean leaves used in the pigment assays are given in table III and figure 4. The increase in fresh leaf weight with irradiance follows the same general pattern as the dry leaf weight increase. It was observed qualitatively that those treatments which were most effective in causing leaf development, inhibition of

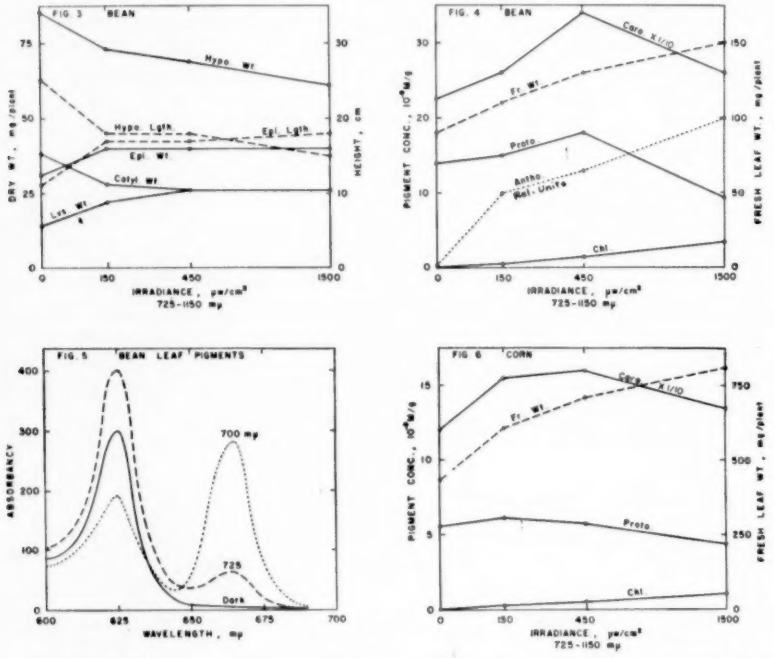

FIG. 3. Influence of irradiance of far red (725 mμ) on the distribution of dry weights and stem lengths of Black Valentine bean after eight days of continuous irradiation. Each determination is for 100 plants.

FIG. 4. Effect of irradiance of far red (725 mμ) on the molar concentrations of bean leaf pigments per gram of fresh weight, hypocotyl anthocyanin, and average leaf fresh weight of bean after eight days of irradiation. Carotene values are 10 times the ordinate scale indicated.

FIG. 5. Transmission curves of ethyl ether extracts of bean leaves. The protochlorophyll maximum appears at 625 mμ and the chlorophyll maximum at 665 mμ. The short wave length limit of the filter used for irradiation is indicated on each curve.

FIG. 6. Effect of irradiance of far red (725 mμ) on the molar concentration of corn leaf pigments per gram of fresh weight and average fresh weight of leaves. Carotene values are 10 times the ordinate scale indicated.

hypocotyl growth, and epicotyl elongation in bean were also the treatments most effective in causing the straightening of the plumular hook. In general, the hook disappeared most rapidly in the red and least rapidly in the blue. At harvest, there was always some evidence of a hook present in the plants in blue irradiance.

Corn (table IV, fig. 6) exhibited an increase in leaf fresh weight, in response to irradiance of 725 mμ, similar to that shown by bean. The red and blue treatments produced the smallest leaves of any of the irradiation treatments in corn as contrasted with bean, although in both treatments the leaves were about 25% heavier than those of the dark controls. In two preliminary experiments with corn, for which the data are not reported, similar results were obtained.

None of the responses reported here can be explained in terms of the thermal effects of the radiant energy. Noon sunlight at 1.4 cal./min./cm.^{2} is equivalent to ca. 100,000 μw./cm.^{2} and the highest far red irradiance used in these experiments, 1500 μw./cm.^{2}, was only 1.5% of this value. A small additional infrared irradiance was produced by the heating of the secondary filters at the highest irradiance, but this did not more than double the irradiance as measured by the water cell and thermocouple detector. Thermometers placed in the plots indicated that there was never more than a 1° C difference in air temperature between compartments.

These results indicate that the central feature of photomorphogenesis in bean is the photochemical initiation of active growth in the epicotyl, directed principally toward the rapid maturation of the leaves. The accelerated terminal growth places greater demands on the cotyledonary food reserves, which results in accelerated translocation to the epicotyl and leaves. In corn the far red accelerates leaf growth, but not to as great a degree as in bean.

TABLE IV

FRESH WEIGHT AND PIGMENT CONTENT OF CORN LEAVES UNDER VARIOUS IRRADIATION CONDITIONS FOR EIGHT DAYS.

Irradiation treatment	Average fresh weight	Pigment per gram fresh weight			
		Carotene	Protochlorophyll (P)	Chlorophyll (C)	P/C
	mg.	μg.	μg.	μg.	
Dark	430	65	4.8	0.00	....
Far red, 725 mμ 450 μw./cm.^{2}	720	87	5.1	0.26	20
Far red, 700 mμ 450 μw./cm.^{2}	720	75	3.1	3.9	0.8
Red, 630–700 mμ 2 μw./cm.^{2}	570	....	....	355	....
Blue, 436 mμ 2 μw./cm.^{2}	570	....	....	200	....

Chlorophyll synthesis

Some chlorophyll was formed in all the treatments with wave length of 725 mμ (figs. 4 and 6). In both bean and corn, the blue wave lengths at 2 μw./cm.^{2} produced approximately 1000 times as much chlorophyll as the 725 mμ wave lengths at 150 μw./cm.^{2} and yet plants from the two plots were comparable in photomorphogenic response. Since some photomorphogenesis occurred at a blue irradiance of 2 μw./cm.^{2}, both the blue and red values were reduced from 2.0 to 0.05 μw./cm.^{2} The results for bean are given in table V. The leaves from the higher irradiance of blue (table III) were 30% heavier than those from the dark, but those from the lower irradiance were only 7% heavier. At the lower irradiance, the plumular hook was present at harvest and the hypocotyl length was only slightly inhibited. In contrast, plants exposed to the far red at 150 μw./cm.^{2} had leaves 23%

TABLE V

FRESH WEIGHT AND PIGMENT CONTENT OF BEAN SEEDLINGS UNDER VARIOUS IRRADIATION CONDITIONS FOR 11 DAYS.

Irradiation treatment	Average fresh weight of leaves	Length of hypocotyl	Leaf pigment per gram fresh weight			Relative concentrations of anthocyanin	
			Carotene	Protochlorophyll	Chlorophyll	Hypocotyl	Cotyledons
	mg.	*cm.*	*μg.*	*μg.*	*μg.*		
Dark	83	25	100	10.0	0.0	6	4,000
Far red, 765 mμ 300 μw./cm.^{2}	85	23	160	15.5	0.0	56	6,100
Far red, 725 mμ 150 μw./cm.^{2}	112	19	165	14.4	0.25	100	6,500
Red, 630–700 mμ .05 μw./cm.^{2}	129	18	....	....	133	95	7,000
Blue, 436 mμ .05 μw./cm.^{2}	89	23	....	....	45	60	5,900

heavier than those in the dark, showing a strong photomorphogenic response, but had only ca. 1/200 of the chlorophyll present in the leaves exposed to the low energy blue wave lengths. The treatment with wave length of 765 mμ (table V) produced a definite increase in leaf weight and a complete loss of plumular hook without the synthesis of any detectable chlorophyll.

While in these experiments strong photomorphogenic responses were not induced without any detectable synthesis of chlorophyll, the complete lack of any correlation between chlorophyll synthesis and photomorphogenesis in the various spectral regions, and the very low levels of chlorophyll formed in the far red treatments, suggest that protochlorophyll and chlorophyll probably do not participate in these photochemical growth reactions. However, until the photoreceptor of the photomorphogenic responses is isolated and characterized, it will be impossible to conclude that minute traces of chlorophyll may not promote certain of these growth responses.

On the basis of experimental considerations, the use of far red radiant energy offers many unique advantages over other regions of the visible spectrum for the excitation of the various growth responses associated with photomorphogenesis. At irradiances of from 150 to 450 μw./cm.^{2}, the amount of chlorophyll formed is for most purposes quantitatively negligible. Photosynthesis is also excluded as a complicating growth factor, either as regards its intermediates or products.

Protochlorophyll and carotene

Tables III and IV show that the two lowest irradiances of 725 mμ caused an increased synthesis of protochlorophyll and carotene per unit fresh weight of leaf tissue over that formed in the dark. Even at 765 mμ, new synthesis of these pigments occurred, although the growth responses were relatively small. In two preliminary experiments with bean and two with corn, low irradiances of 725 mμ produced similar results. Since the leaves of irradiated plants were always heavier than those of the plants grown in the dark there was a still greater pigment increase per plant. Both protochlorophyll and carotene increased with irradiance at 725 mμ to a maximum value and then fell with further increase in irradiance (figs. 4 and 6). At the highest irradiance, the protochlorophyll fell below the value for both bean and corn in the dark, but the chlorophyll concentration increased with increasing irradiance. Chlorophyll synthesis, however, could account for only a small proportion of the loss in protochlorophyll and carotene. For each additional molecule of chlorophyll formed in bean by 1500 μw./cm.^{2} above that formed by the 450 μw./cm.^{2} treatment, there was an approximate loss of 4 molecules of protochlorophyll and 40 of carotene. In corn, the relative protochlorophyll decrease was 3, while the carotene decrease was 50 for each new chlorophyll molecule formed.

Figure 5 shows how the absorption spectra of the leaf pigment extracts from 600 to 700 mμ changed when the plants were irradiated with wave lengths of 700 mμ and 725 mμ respectively at 450 μw./cm.^{2} The spectrum for the extract of the plants grown in the dark contains only the absorption maximum for protochlorophyll at 625 mμ. Irradiation with 725 mμ caused both an increase in protochlorophyll and the appearance of a small chlorophyll maximum at 665 mμ, but at 700 mμ, sufficient chlorophyll synthesis occurred to depress the protochlorophyll level. Since the two low irradiances at 725 mμ caused the synthesis of only traces of chlorophyll, it is evident that the observed increases in protochlorophyll and carotene could not have been caused by products arising from photosynthesis. It appears more than likely that the increased synthesis of these pigments was the indirect result of photomorphogenesis.

The rise and subsequent fall in protochlorophyll and carotene with increasing irradiance followed a course very similar to that observed by Frank (**5**) for carotene in Avena coleoptiles. Frank speculated that the fall in carotene concentration was due to the diversion of carotene precursors into those early steps of chlorophyll synthesis where the phytol group is

added onto a porphyrin nucleus. This interpretation is difficult to reconcile with the results reported here in which the drop in molar concentration of carotene is of the order of 50 times the molar increase in chlorophyll. Even on a per plant basis where the leaves were increasing in size, the concentration of both carotene and protochlorophyll increased and then decreased. This indicates that the rise and fall in concentration of these two pigments with irradiance was the result of interacting factors controlling synthesis and decomposition which were not directly related to the biosynthesis of chlorophyll precursors.

Anthocyanin synthesis

Dark-grown seedlings of Black Valentine bean contain very high concentrations of anthocyanin in the cotyledons. Traces of purple color are visible at the cotyledonary node and in the leaf veins, but all other portions of the plant appear either white or yellow. After an exposure of eight days to the longer wave lengths of the visible spectrum, additional anthocyanin occurs in the hypocotyl and epicotyl, the most intense coloration appearing in the region of the cotyledonary node. It was observed that the corn seedlings were devoid of any visible purple coloration when grown in the dark, but the leaf bases became pink when exposed to red or far red energy.

The cotyledons of plants grown in the dark (tables III and V) contained a high concentration of anthocyanin per unit fresh weight, but the concentration of anthocyanin increased nearly 50% following treatment with the higher irradiances of far red. The hypocotyl concentration increased about 20-fold with the 1500 μw./cm.^{2}–725 mμ treatment. Even at 765 mμ, where the photomorphogenic response was small as measured by increase in leaf weight and inhibition of the hypocotyl, the anthocyanin increased about 50% in the cotyledons and almost 10-fold in the hypocotyl. The curve of relative anthocyanin in bean hypocotyl at various irradiances at 725 mμ shows that, unlike carotene and protochlorophyll, anthocyanin increased with increasing irradiance over the range used. The anthocyanin concentration closely paralleled the photomorphogenic response as measured by leaf fresh weight increase.

While several reports (**3, 12**) have demonstrated that low temperature and sugars supplied in the substrate or endogenously via photosynthesis will promote the synthesis of anthocyanin, it is evident that these are not the factors contributing to synthesis by far red radiation, as reported here. Whether the increased synthesis of anthocyanin with radiant energy is a direct or indirect photo-response is not clear as yet.

Summary

The design of a growth chamber and a water-cooled and filtered irradiation system is described for growing plants under controlled conditions of temperature, humidity, irradiation, and nutrition.

Results are presented on the effects of the following spectral regions, applied continuously, on seedling growth and pigment synthesis in bean and

corn: the blue 436 mμ mercury line, red from 630 to 700 mμ, and three far red bands of 700 to 1150 mμ, 725 to 1150 mμ, and 765 to 1150 mμ. Maximum photomorphogenesis occurred with wave lengths of 630 to 700 mμ.

Irradiances at 725 mμ of 150 to 450 μw./cm.^{2} are capable of inducing a strong photomorphogenic response with synthesis of only traces of chlorophyll. However, no combination of far red energy was found in this series of studies which induced a strong photomorphogenic response with no detectable chlorophyll. At 765 mμ, a weak photomorphogenic response was obtained with no measurable formation of chlorophyll.

Blue irradiance of 2 μw./cm.^{2} induced weak photomorphogenesis with a chlorophyll concentration about 1000-fold greater than that in plants under wave lengths of 725 mμ with an equal degree of development. From these data, it is concluded that there is no quantitative relationship between chlorophyll synthesis and the photomorphogenic responses.

Data are presented on the effect of 725 mμ irradiances at 0, 150, 450, and 1500 μw./cm.^{2} on dry weight distribution, stem lengths, and anthocyanin content in the bean plant, and fresh leaf weight, chlorophyll, protochlorophyll, and carotene in bean and corn. In both species, fresh leaf weight increased with increase in irradiance. Protochlorophyll and carotene synthesis were increased by low irradiances over the synthesis in the dark, but decreased with further increasing irradiances. All irradiation treatments used caused large increases in anthocyanin in bean hypocotyl. At 725 mμ the anthocyanin progressively increased with increase in irradiance. The synthesis of anthocyanin in bean and corn is dependent upon a photo-process which does not involve photosynthesis, as shown by marked formation of anthocyanin in the far red where photosynthesis did not occur.

Appreciation is extended to Dr. V. Schocken who collaborated on certain experiments preliminary to those reported in this paper. This work was supported in part by a grant from the Atomic Energy Commission under Contract AT(30-1)-1133. The authors wish to express their sincere appreciation to the Research Corporation for a grant which provided many of the facilities used in this research.

LITERATURE CITED

1. Bandurski, R. S. Synthesis of carotenoid pigments in detached bean leaves. Bot. Gaz. **111**: 95–109. 1949.
2. Chen, S. L. The action spectrum for the photochemical evolution of oxygen by isolated chloroplasts. Plant Physiol. **27**: 35–48. 1952.
3. Eddy, B. P. and Mapson, L. W. Some factors affecting anthocyanin synthesis in cress seedlings. Biochem. Jour. **49**: 694–699. 1951.
4. Emerson, R. and Lewis, C. M. The dependence of the quantum yield of Chlorella photosynthesis on wavelength of light. Amer. Jour. Bot. **30**: 165–178. 1943.
5. Frank, Sylvia. The relation between carotenoid and chlorophyll pigments in Avena coleoptiles. Arch. Biochem. **30**: 52–61. 1951.

6. Gibson, K. S. Spectrophotometry. Nat. Bur. Standards Cir. **484**. 1949.
7. Goodwin, R. H. and Owens, Olga v. H. The effectiveness of the spectrum in Avena internode inhibition. Bull. Torrey Bot. Club **78**: 11–21. 1951.
8. Koski, Violet M. Chlorophyll formation in seedlings of *Zea mays* L. Arch. Biochem. **29**: 339–343. 1950.
9. Koski, Violet M., French, C. S., and Smith, J. H. C. The action spectrum for the transformation of protochlorophyll to chlorophyll a in normal and albino corn seedlings. Arch. Biochem. Biophys. **31**: 1–17. 1951.
10. Parker, M. W., Hendricks, S. B., Borthwick, H. A., and Went, F. W. Spectral sensitivities for leaf and stem growth of etiolated pea seedlings and their similarity to action spectra for photoperiodism. Amer. Jour. Bot. **36**: 194–204. 1949.
11. Schneider, C. L. The effect of red light on growth of the Avena seedling with special reference to the first internode. Amer. Jour. Bot. **28**: 878–886. 1941.
12. Thimann, K. V. and Edmondson, Yvette H. The biogenesis of the anthocyanins. I. General nutritional conditions leading to anthocyanin formation. Arch. Biochem. **22**: 33–53. 1949.
13. Weintraub, R. L. and Price, L. Developmental physiology of the grass seedling. II. Inhibition of mesocotyl elongation in various grasses by red and violet light. Smithsonian Misc. Coll. 106(21): 1–15. 1947.
14. Whitmore, R. A. Light and pigment development in the kidney bean. Plant Physiol. **19**: 569–578. 1944.
15. Withrow, R. B. Response of seedlings to various wave bands of low intensity irradiation. Plant Physiol. **16**: 241–256. 1941.
16. Withrow, R. B. and Elstad, V. Water-cooled lamp systems containing self-condensing aqueous light filters. (In press.)
17. Withrow, R. B. and Price, L. Filters for the isolation of narrow regions in the visible and near ultraviolet spectrum. Plant Physiol. **28**: 105–114. 1953.
18. Withrow, R. B. and Withrow, Alice P. Nutriculture. Purdue Univ. Agr. Exp. Sta. Cir. 328. 1948.
19. Zscheile, F. P., White, J. W., Jr., Beadle, B. W., and Roach, J. R. The preparation and absorption spectra of five pure carotenoid pigments. Plant Physiol. **17**: 331–346. 1942.

STUDIES ON THE CHEMISTRY OF THE LIVING BARK OF THE BLACK LOCUST IN RELATION TO ITS FROST HARDINESS. III. THE VALIDITY OF PLASMOLYSIS AND DESICCATION TESTS FOR DETERMINING THE FROST HARDINESS OF BARK TISSUE[1]

D. SIMINOVITCH[2] AND D. R. BRIGGS

DIVISION OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA, ST. PAUL, MINNESOTA

Received June 5, 1952

Cellular tests which are used in place of actual freezing tests to estimate the resistance to injury by freezing that a plant tissue will show are based on the observations of LEVITT and SCARTH (**2, 5**) and LEVITT, SCARTH and SIMINOVITCH (**3, 6, 7, 10**) that the most characteristic physical property of frost hardy cells is their capacity to withstand injury by dehydration.

The frost hardiness test described in the first papers of the present series (**1, 11**) was based on a determination of the percentage survival of cells after dehydration by plasmolysis, as effected by subjecting the tissues to a graded series of strong salt solutions. In subsequent studies hardiness has been determined also by estimating the survival of the cells after desiccation in atmospheres of varied relative humidities produced over a graded series of sulphuric acid solutions. The degree of hardiness has been assumed to be measurable by the extent to which the cells possess the property of surviving dehydration. Experimental verification of the validity of this assumption by comparison of dehydration tests with actual freezing tests has been obtained previously (**6, 9**) with other plant tissues but not with the bark cells of the black locust, the experimental tissue employed in these studies.

Dehydration tests for frost hardiness have tremendous advantages over actual freezing tests with regard to the time required to make a test, the amount of test material needed, and the cost of the equipment involved. In studies such as those of the present series, where a large number of individual determinations of hardiness is required, and where often only a limited amount of test material is available, freezing tests cannot be used effectively. It is important, therefore, to establish the validity of dehydration tests when applied to the bark tissues of black locust.

Materials and methods

TEST FOR RESISTANCE TO PLASMOLYSIS INJURY

Samples of living bark for testing resistance to plasmolysis injury were obtained by removing whole bark from the trunk in one-inch squares. The

[1] Paper no. 2844, Scientific Journal Series, Minnesota Agricultural Experiment Station.

[2] Present address: Division of Chemistry, Department of Agriculture, Science Service, Ottawa, Canada.

outer dead bark was pared off to expose the outer face of the living bark. A number of thin tangential sections were sliced from the face of the bark with a sharp razor blade, and a group of 10 sections of tissue was transferred to each of a graded series of balanced salt solutions ($NaCl : CaCl_2 :: 9 : 1$) of increasing concentration (0.5 molar to 5.0 molar). The sections were plasmolyzed in these solutions for 10 minutes, and transferred to fresh tap water for five minutes to deplasmolyze. Excess water was removed and the tissues stained with neutral red (a few drops of a 1% solution to 20 cc. of H_2O). After a few minutes in the staining solution, the sections were again transferred to fresh tap water for 10 minutes and then examined under the microscope. Only those cells which absorbed and retained neutral red were considered to have survived the plasmolysis and deplasmolysis process. Since 10 sections, each containing at least 100 cells, were examined after treatment in each plasmolyzing solution, the estimates of survival were based on at least 1000 cells.

The length of exposure of the sections in the salt solutions and the time permitted to elapse before the percentage survival was estimated were arbitrarily chosen. Protracted exposure to the plasmolyzing salt solutions will increase the amount of injury obtained. Similarly, if the appraisal of the percentage of survival in the tissues after staining and transfer to water is delayed, smaller survival values are obtained. These variations possibly arise from toxic effects of the dye and of the salts in the plasmolyzing solution. The optimal time interval for immersion of the stained sections in water after staining can be determined by a study of the time required for toxicity of neutral red to manifest itself in cells not previously exposed to plasmolyzing conditions. Where such precautions are taken to avoid toxic effects due to the dye itself, comparisons of estimates of survival based on staining by neutral red with estimates based on visual examinations of browning, leaching, etc., have shown staining and retention of neutral red in cells to be a reliable index to the viability of these cells. Staining alone, without subsequent immersion in water for a period of time, is not sufficient as a criterion of survival because accumulation of dye is observed to occur in partially injured cells. After transfer to water, the dye leaches out of these cells more rapidly than from healthy cells.

Test for resistance to desiccation injury

Solutions of sulphuric acid of varying concentrations were prepared according to the relative humidities desired and placed in a series of screw-top jars. Tangential sections of the living bark, cut in the same manner as for the plasmolysis tests, were placed in waxed cups suspended from hooks on the covers of the jars so that the cups would hang in close proximity to the surface of the sulphuric acid solutions when the covers were screwed tightly into place. Rapid dehydration of the cells occurred, and after 24 hours at room temperature, the vapor pressure of the tissues was in approximate equilibrium with the sulphuric acid solutions.

The wax cups were lifted out of the jars and wetted directly with water to saturate the tissue slices. The tissues were then transferred to tap water, stained with neutral red, again immersed in fresh tap water and the percentage survival of the cells after exposure to each of the desiccating atmospheres determined as described for the plasmolysis tests.

Test for resistance to freezing injury

To provide conditions as natural as possible for the freezing process within the bark tissues, sections of whole living bark, with the immediately adjoining corky bark and wood still adhering, were used for the freezing tests. Three-inch lengths of logs complete with bark were cut from the trunks of trees and wedge-shaped sections were split from these lengths by a number of longitudinal cuts in a radial direction. Most of the wood was then split off each section by a tangential cut at a distance of 3 millimeters from the cambial layer. After trimming off some corky bark on the outside, the sections were one inch in width, about three fourths inch in thickness and three inches in length and contained the whole living bark between a thin layer of corky bark and sap wood. In this manner a sample of whole bark adequate for the freezing test was obtained without departing too radically from the conditions surrounding the bark in the intact tree. The small size of the samples permitted the simultaneous testing of samples from many different trees within the limited space available in the testing compartment of the freezing unit. At the time of a freezing test these sections, cut from different trees, were distributed into mason jars which were then spaced in the compartment to allow free movement of the circulating air.

The freezing unit used was a commercially available apparatus (American Instrument Co.) consisting of a large, well insulated cabinet divided by a similarly insulated partition into two compartments. Dry ice is placed in one compartment while the other, of 8 cubic feet capacity, functions as the test compartment. The two chambers are connected through a duct with a hand-operated damper which can be used to vary the rate of flow of air from one chamber into the other. Temperature control is achieved by a manually adjusted bimetallic thermo-regulator and a sensitive relay which activates, either separately or together, a blower and a heating element which is mounted in the test compartment. The blower forces air over the dry ice and into the test compartment for cooling. The heater or blower can be operated independently. In the very low temperature range (below $-15°$ C) only the blower is used, and the temperature range is regulated by its intermittent action. For close temperature control above $-15°$ C the heater is operated in opposition to the cooling system. A continuously operating fan maintains circulation of air within the test compartment. By manual adjustment of the thermo-regulator and damper and an adequate supply of dry ice, any desired temperature between 0 and $-70°$ C can be maintained and controlled within one degree for an indefinite period of time. With the heating circuit in operation at temperatures above $-15°$ C, 40 lb. of dry ice

is required every 24 hours. Less dry ice is required at lower temperatures after the chamber has once reached equilibrium. The chambers are so well insulated that the blower operates for brief intervals only even at temperatures of – 70° C. This apparatus made it possible to reproduce easily any conditions of extremes or variations in temperature to be found in cultivated areas of northern latitudes. However, some arbitrary rate of drop in temperature had to be selected. To avoid killing due to freezing of an intracellular nature which might be incurred with too rapid a drop in temperature or with excessive supercooling, the following procedure was adopted. The test material was placed in the cabinet at room temperature, and the temperature was then lowered rapidly to just above the freezing point and maintained there for several hours until the tissues had reached equilibrium. The temperature of the cabinet was then lowered at the rate of 1° C per hour. The response in temperature drop was instantaneous but the selected rate of drop in temperature permitted the temperature of the tissue to come to equilibrium with that of the circulating air before it was lowered another increment. The temperature inside the test compartment was read through a plate glass window in the cover of the cabinet from a thermometer mounted inside the cabinet. The temperature of the tissue was measured by a thermo-couple inserted into the center of a representative sample and connected to a galvanometer circuit on the outside. When each of the various freezing temperatures required for the test was reached in turn, mason jars containing samples from each of the trees being tested were removed for thawing.

Several of the samples in each test were held at – 1° C for 24 hours to thaw slowly. Where rapid thawing was desired, the samples were plunged immediately into tap water at room temperature. After slow or rapid thawing, transverse or tangential sections were cut from the living bark, and the percentage of cell survival was determined by the neutral red staining technique already described.

The large samples used in the freezing test permitted survival to be estimated in other ways. After thawing, the corky bark and wood were removed and the samples were immersed again in tap water for an hour. At the end of this period any freezing injury could be detected easily by observing the degree of flaccidity of the tissue and the extent of leaching and browning. The percentage of survival determined in this way always paralleled that observed in the staining test.

A BASIS FOR COMPARISON OF RESISTANCE TO INJURY BY THE VARIOUS METHODS

If freezing injury in locust bark cells results primarily from dehydration of the cells due to diffusion of water from the cellular fluids to extracellular ice crystals as freezing progresses, the survival achieved, under any of the tests described, can be regarded as a measure of the capacity of the tissue to withstand such dehydration. A comparison of the various tests can be made

on the basis of the dehydration intensity which in each test is accompanied by an equal lethal effect on the tissue. For equal degrees of dehydration intensities imposed, comparable dehydration will occur when the relative vapor pressure, P/Po (where Po = vapor pressure of pure water and P = vapor pressure of the water in the cell at equilibrium), of the water in the cell is brought to the same value under each type of test applied. Since the difference in the partial molar free energy of a solvent is equal to RT ln Po/P

TABLE I

RELATIVE VAPOR PRESSURES, MOLARITIES, AND FREEZING POINT DEPRESSIONS EQUIVALENT TO VARIOUS DEHYDRATION INTENSITIES EMPLOYED IN THE DESICCATION, PLASMOLYSIS, AND FREEZING TESTS, RESPECTIVELY.

Dehydration intensity $\text{Log } \frac{P_o}{P}$	Relative vapor pressure $\frac{P}{P_o}$	Molarity of solutions	Freezing point depression	H_2SO_4*	Osmotic pressure
			°C	*wt. %*	*atmos.*
0	1.00	0	0	0	0
0.0086	0.98	0.62	2.05	3.6	24.8
0.0179	0.96	1.18	4.26	7.4	51.5
0.0269	0.94	1.72	6.43	10.9	77.4
0.0362	0.92	2.25	8.65	14.0	104.2
0.0457	0.90	2.77	10.91	16.9	131.5
0.0554	0.88	3.26	13.22	19.5	159.5
0.0652	0.86	3.75	15.56	21.2	187.7
0.0755	0.84	4.19	18.03	23.0	217.1
0.0864	0.82	4.61	20.61	25.7	248.6
0.0969	0.80	5.02	23.13	26.3	278.8
0.1248	0.75		29.79	30.1	359.5
0.1547	0.70		36.93	33.2	445.0
0.1870	0.65		44.62	35.9	538.0
0.2219	0.60		52.97	38.4	638.5
0.2596	0.55		61.95	40.8	747.0

*Giving corresponding relative vapor pressure.

(temperature held constant) and since the value of P/Po is virtually independent of temperature, the value of log Po/P can be taken as a measure of the dehydration intensity, and serve as a basis for comparison.

In the desiccation test, the relative vapor pressure of the water remaining in the tissue is obtained directly from the relative humidities of the sulphuric acid solutions employed. The relative vapor pressure of water in equilibrium with ice at various temperatures below 0° C can be calculated from the equation, $\log Po/P = 0.00419\,\Delta T$, where P/Po = the relative vapor pressure and ΔT = the temperature in degrees centigrade below 0° C, to which the tissue is subjected in the freezing test. The relative vapor pressures of the solutions employed in the plasmolysis tests can be similarly calculated from the freezing points of solutions of NaCl and of $CaCl_2$ (Inter-

national Critical Tables, Volume IV, pages 257–258) and the assumption that the effect of each salt is additive in the mixtures here employed.

The conditions of dehydration which result in a standard degree of killing of the cells of the tissue sample are compared. The dehydration intensity (log Po/P) required with each sample to effect a 50% kill of the cells under one type of test is plotted against the corresponding dehydration intensity required by a second type of test to effect an equal degree of killing. In table I are given the relative vapor pressures (P/Po), the molarity of $NaCl:CaCl_2$ solutions, and the freezing point depressions (ΔT) equivalent to various dehydration intensities (log Po/P) relating to the conditions employed in the desiccation, plasmolysis and freezing tests, respectively.

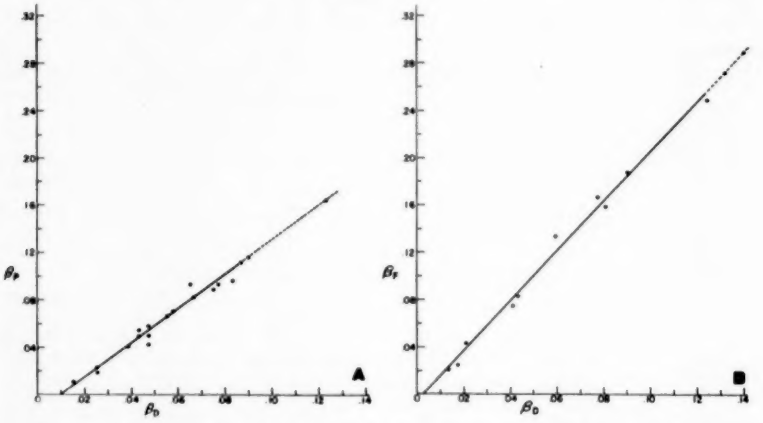

FIG. 1. A. Comparison of dehydration intensities, log Po/P, required to effect a 50% killing of bark cells in the plasmolysis and desiccation tests. β_P = dehydration intensity in the plasmolysis test, β_D = dehydration intensity in the desiccation test. B. Comparison of dehydration intensities, log Po/P, required to effect a 50% killing of bark cells in the desiccation and freezing tests. β_D = dehydration intensity in the desiccation test, β_F = dehydration intensity in the freezing test.

The concentration of sulphuric acid solutions which yield the corresponding relative vapor pressures in the desiccation tests and the calculated values of osmotic pressure (at 0° C) corresponding to the various dehydration intensities are also included.

Results

The extent to which the resistance of the bark tissues to injury by plasmolysis may be correlated with their resistance to injury by desiccation was first examined. To study this relationship over a wide range, a series of bark tissue samples which had previously been found to differ markedly in their resistance to injury by plasmolysis were tested simultaneously for resistance to injury by both processes. The relation of plasmolysis resistance to desiccation resistance is shown graphically in figure 1 A as the values of

dehydration intensity required by each method to effect 50% killing of the cells of each of the samples tested.

The points in figure 1 A beyond that position which corresponds to a solution of 5 molar concentration (or a plasmolyzing dehydration intensity equivalent to 0.096) represent the results of desiccation tests on samples of bark tissue obtained from trees in early and late winter. At these times the frost resistance is at or near a maximum, and the tissues show extreme resistance to injury by plasmolysis. Even immersion in the 5 molar solution is not sufficient to produce 50% killing of their cells. Since a 5 molar solution approximates the highest concentration of balanced salt solution attainable at ordinary temperatures, resistance to injury from plasmolysis could not be measured in these tissues. On the other hand, no degree of resistance to dehydration was found in the cells of tissues of the black locust even in winter which could not be measured by means of the desiccation tests, since the dehydration intensity of the desiccating atmospheres which are attainable over even moderately concentrated sulphuric acid solutions readily exceeds this limit. The dehydration intensities which were required in desiccation tests for 50% killing of the cells in those samples whose resistance could not be measured by the plasmolytic method are indicated in figure 1 A by circles placed on the dotted extrapolation of the curve defining the relationship between the two tests.

The time of exposure of the tissues to the concentrated solutions used in the plasmolysis test was limited to 10 minutes. Prolonged immersion of these tissues in the solutions leads to a gradual increase in the number of cells killed. Even the cells of the most resistant tissues eventually succumb. It would appear, then, that the resistance to injury from plasmolysis of even the most resistant tissues might be measured under such conditions. However, protracted plasmolysis is undesirable because the toxic effects of the salt solutions influence the results in addition to the effects of dehydration. Nevertheless, it is probable that, with another choice of plasmolytic agents (*e.g.*, sugars) and with an increase in plasmolysis time, the range of the plasmolysis test could be extended considerably.

From figure 1 A it is evident that, within the limits of the plasmolysis tests, the tissue resistance to plasmolysis injury is definitely and linearly related to its resistance to desiccation injury. While a few points deviate considerably from the regression curve, this situation could undoubtedly be improved by stricter adherence to the empirically chosen procedures followed in each of the tests. The significance of the failure of the regression line to pass through the origin is unknown but may stem from the toxic effects of the plasmolysis solutions which do not vary necessarily in the same manner as their dehydration intensities.

It remained to be shown that one or the other form of resistance could be similarly correlated with resistance to freezing injury. That a relationship exists between resistance to injury by plasmolysis and actual hardiness to winter conditions has been repeatedly indicated. This relationship may be

illustrated by a series of ringing experiments performed on two locust trees. At different times during a summer these trees were freshly bark ringed, the new rings being placed on each tree at progressively higher levels. Assuming that an estimate of resistance to injury by plasmolysis would furnish some measure of their frost resistance, plasmolysis tests were performed in October on the various segments of the bark of these trees. These tests indicated that the bark of those sections of the trunks between rings which were situated lowest on the trunk, and which therefore had been intercepted

TABLE II

THE RELATIONSHIP OF THE RESISTANCE OF THE BARK TISSUES OF RINGED LOCUST TREES TO PLASMOLYSIS INJURY AND THEIR CAPACITY FOR WITHSTANDING NATURAL FREEZING CONDITIONS DURING THE WINTER.

Ringed tree	Ring	Time of ringing	Part of tree	Per cent. survival of tissues after exposure to molarities of plasmolyzing solutions, October test					Per cent. survival of tissues after exposure to natural freezing conditions during winter, March estimate
				1M	2M	3M	4M	5M	
1			Crown, above ring 5	100	100	100	100	100	100
	5	September 23	Between ring 4 & 5	....	....	....	....	....	100
	4	September 3	Between ring 3 & 4	100	100	100	70	30	100
	3	August 18	Between ring 2 & 3	100	100	100	50	10	30
	2	August 2	Between ring 1 & 2	100	100	80	30	0	0
	1	August 2	Base, below ring 1	100	100	100	100	50	90
2			Crown, above ring 5	100	100	100	100	50	100
	5	September 23	Between ring 4 & 5	....	....	....	....	....	....
	4	September 3	Between ring 3 & 4	100	100	100	95	30	90
	3	August 18	Between ring 2 & 3	....	....	....	....	....	30
	2	August 11	Between ring 1 & 2	100	100	90	50	10	10
	1	August 11	Base, below ring 1	100	100	100	100	80	100

earliest during the summer, had developed considerably less resistance to plasmolysis injury than the bark of normal intact trees at this time. The resistance of those sections located between the earlier rings was also less than that of the sections between later rings and considerably less than those of the terminal or basal parts of the same tree above and below the rings. A considerable range of hardiness as measured by resistance to plasmolysis injury was therefore encountered in the different parts. These differences in hardiness were so marked that the results of the plasmolysis tests were checked with actual freezing tests. The ringed trees were left standing and exposed to natural conditions for the duration of the winter. The fol-

lowing spring the extent of survival of the various sections was appraised and found to correspond very closely to that expected from the results of the plasmolysis tests in October. These results are shown in table II. The actual degrees of hardiness and resistance to injury from plasmolysis which were reached finally in mid-winter in all parts of these ringed trees were probably greater than those indicated in October, but the increase was nevertheless not sufficient to enable the bark of some of these sections to survive winter conditions. The plasmolysis tests performed in the fall served quite adequately to indicate the capacity of these various tissues to withstand freezing conditions during the winter.

Because a freezing test performed under natural conditions measures the effect of the sum total of a large variety of conditions which are not easily reproducible from year to year and are not amenable to quantitative expression, a dependable basis for the evaluation of dehydration tests could be arrived at only by comparing them with freezing tests performed under controlled conditions in the laboratory. Having shown the existence of a direct relationship between the plasmolysis and desiccation tests and because of the greater range afforded by the latter, the desiccation test was adopted in preference to the plasmolysis test for quantitative comparisons with the controlled freezing tests. Freezing tests (with slow thawing) and desiccation tests were performed simultaneously on tissues of each of a series of bark samples taken from trees at different stages of hardiness. The results of these tests are presented graphically in figure 1 B where the dehydration intensities required to produce killing of 50% of the cells in desiccation tests are plotted against the dehydration intensities which correspond to the temperatures to which the samples had to be frozen in order to obtain the same degree of killing.

Over a considerable range of tissue hardiness, the resistance or susceptibility of cells to injury by desiccation is shown to be linearly proportional to their resistance or susceptibility to injury by freezing. In view of the previously demonstrated correlation of plasmolysis resistance with desiccation resistance, a similar linear proportionality must exist between plasmolysis resistance and freezing resistance.

Equations describing the regression lines statistically derived from the data in figures 1 A and B, respectively, are $\beta_D = 0.696\ \beta_P + 0.010$ ($r = 0.980$) and $\beta_D = 0.464\ \beta_F + 0.003$ ($r = 0.994$), where the symbols β_D, β_P, and β_F represent the dehydration intensities of desiccation, plasmolysis and freezing conditions of the various tests employed (r is the regression coefficient). From these equations the relationship between the plasmolysis and freezing tests may be calculated as $\beta_P = 0.667\ \beta_F + 0.010$.

None of these tests is exactly equivalent to any of the others, *i.e.*, the slope of the curve describing their interrelationship in no case is equal to one, which must be interpreted to mean that the dehydration intensity is not the sole determining factor in the killing process under the tests as run. All of the tests are empirical as to procedure, other than the conditions of dehy-

dration intensity imposed, and the choice of 50% killing as the level for comparison is arbitrary. That conditions other than intensity of dehydration are involved in determining the extent of killing is illustrated also, when the percentage survivals of the cells of an identical tissue are compared after samples are subjected to varying intensities of dehydration by each of the three methods of testing. Figure 2 shows this relationship where a tissue of an intermediate degree of hardiness was studied by all three methods. The importance of an empirical difference in procedure, when introduced into a given test, is shown in this graph by the change which results in the percentage survival when slow as compared to fast thawing procedures are followed in the freezing test. It is also evident that those tests conducted at room temperature (plasmolysis and desiccation) are sharper in their killing effects than is the freezing test itself; that is, a wider range of dehydration intensities is required at low temperatures to span the

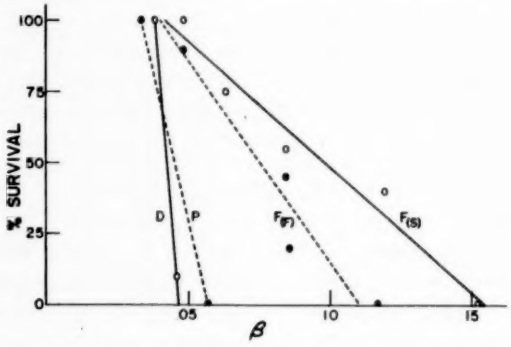

FIG. 2. Per cent. survival *versus* dehydration intensity, β, for a tissue of moderate hardiness under conditions of the plasmolysis (P), desiccation (D), freezing with fast thawing $F_{(F)}$ and freezing with slow thawing $F_{(S)}$ tests.

killing range from zero to 100% than is required at the higher temperatures. In this regard, the desiccation test is sharper in its action than is the plasmolysis test. Because of this circumstance alone, it is apparent that comparison at 100% survival instead of 50% survival would tend to yield ratios (slopes in such comparisons as those in Fig. 1 A and B) closer to unity, *i.e.*, a near equivalence of the killing effects under equal dehydration intensities. Such equivalence is, however, of no real importance since any freezing test that might be applied must itself be empirical and relative in comparison with the variable climatic conditions which may exist during any winter season. The point of paramount importance derived from the relationships shown in figure 1 is that the killing capacities of all of these tests are closely proportional to one another when compared on the basis of dehydration intensities and that any one of them will yield a dependable comparison of the capacity of a series of tissues to withstand injury by freezing.

TABLE III

THE RELATIONSHIP OF THE RESISTANCE OF THE BARK TISSUE OF RINGED LOCUST TREES TO DESICCATION INJURY AND CAPACITY FOR WITHSTANDING INJURY BY FREEZING UNDER NATURAL AND CONTROLLED FREEZING TEMPERATURES.

Ringed tree	Ring	Time of ringing	Part of tree	Per cent. survival of tissues after exposure to desiccating atmospheres of relative humidity indicated, tests made in November									Per cent. survival of tissues after exposure to freezing conditions indicated, tests and estimates made in March				
													Natural winter conditions	Controlled freezing temperatures			
														Slow thawing			Rapid thawing
				89	88	86	84	83	81	80	74	69		−32° C	−40° C	−60° C	−60° C
1B			Crown	100	100	100	100	100	40	35	0	0	100	95	95	95	70
	4	August 18															
	3	July 27	3	100	100	100	100	100	50	30	0	0	100	80	80	60	20
	2	July 7	2	100	100	100	50	0	0	0	0	0	60	60	50	20	0
	1	July 7	1	80	20	0	0	0	0	0	0	0	0	....	....	....	....
			Base	100	100	100	100	50	30	30	10	0	100	100	100	100	30
4R			Crown	100	100	100	100	50	80	40	20	30	100	100	100	100	100
	5	September 21															
	4	September 2	4	100	100	100	100	80	80	30	0	0	100	100	100	100	100
	3	August 24	3	100	100	100	100	80	30	0	0	0	100	100	100	80	60
	2	August 15	2	100	100	100	20	10	0	0	0	0	100	100	100	80	20
	1	August 15	1	100	40	50	0	0	0	0	0	0	70	60	50	0	0
			Base	100	100	100	100	40	50	40	10	0	100	100	100	100	30
5R			Crown	100	100	100	100	80	60	70	35	10	100	100	100	100	100
	4	September 9															
	3	August 24	3	100	100	100	100	80	70	30	20	30	100	100	100	100	100
	2	August 15	2	100	100	100	100	40	20	0	0	0	100	100	100	95	25
	1	August 15	1	100	100	60	10	0	0	0	0	0	100	50	60	60	40
			Base	100	100	100	90	80	20	40	10	0	100	100	100	100	40

In table II, it was shown that plasmolysis tests, made in October, provided a useful index to the relative survival capacity of bark tissues of locust under natural freezing conditions of the following winter. In table III, the results from a group of similar experiments illustrate that desiccation tests made in November provide also a reliable index to survival capacity. The desiccation test provides a greater range also than the plasmolysis test. As in the earlier experiments, several trees were ringed at different times during the summer at progressively higher levels along the trunk. The hardiness of the bark of these tree segments was tested in November by means of the desiccation test. A wide range of resistance to injury by desiccation was detected, the bark of the early ringed sections showing, again, the least resistance to injury from dehydration. In November, the trees were cut into log sections at the points of ringing and placed outdoors in close proximity to the laboratory building for the duration of the winter. In the spring it was found, as in the previous ringing experiments, that the tissues with the least resistance to desiccation injury in the fall had not survived. However, much less injury was encountered in the remaining sections than had been expected on the basis of the desiccation tests or on the basis of the degree of survival observed in the earlier ringing experiment where the trees had been left standing during the winter. It was surmised that temperature conditions outdoors in the storage location of the log sections had not been severe enough to cause killing, which was a fortunate circumstance because the tissues of the surviving sections could now be retested under conditions of the controlled freezing test in the laboratory. The differences in hardiness between the tissues of the various sections as predicted on the basis of desiccation tests were confirmed in these freezing tests as is illustrated in table III, where the values of resistance to desiccation injury found in the various ringed sections are compared with the degree of survival observed in these sections under outdoor conditions as well as after being subjected to low temperatures in the controlled freezing tests. In order to confirm the existence of differences in hardiness between the most hardy tissues as they were indicated by the desiccation tests but which could not be revealed by freezing at $-60°$ C with slow thawing, the result of one test at $-60°$ C with rapid thawing is included in the table.

Resistance to desiccation injury was determined on these tissues in November while resistance to freezing injury was determined the following March. As indicated previously, further changes in hardiness often take place in trees during this period, and these changes do not occur to exactly the same degree in different trees. For this reason it cannot be expected that, in these ringed trees, the resistance to freezing injury would show the same correlation with resistance to desiccation injury as was found in the course of those experiments based on the results of controlled freezing and desiccation tests performed simultaneously (fig. 1 B). A broad parallelism nevertheless exists between the resistances to freezing predicted from desiccation tests made in November and those observed by actual freezing tests on tissues from the same source in March.

Results of plasmolysis, desiccation and freezing tests performed simultaneously on samples of bark tissue taken from the same tree in mid-summer and in mid-winter are of interest and are given in table IV. They illustrate the extreme resistance to injury by dehydration or freezing which is developed in winter in this tissue in contrast to the small degree of resistance found in the summer tissue. This extreme tolerance of bark tissue

TABLE IV

A COMPARISON OF THE BARK TISSUES OF THE SAME TREE IN THE WINTER AND IN SUMMER WITH RESPECT TO THEIR PROPERTIES OF RESISTANCE TO PLASMOLYSIS, DESICCATION, AND FREEZING.

Date of collection of sample for testing	Concentration of plasmolyzing solution giving 50% killing	Relative humidity of desiccating atmosphere giving 50% killing	Freezing temperature (slow thawing) giving 50% killing
			°C
February 6	5M	64	−60
July 28	1M	96	−5

in winter to the effects of dehydration is of particular significance in view of the current and widely accepted practice of watering trees in fall to avoid injury by winter desiccation. It seems improbable that under normal or even adverse conditions sufficient water is ever lost from trees in winter to produce that degree of desiccation which is required to kill their cells when in the frost-hardened condition.

Discussion

The foregoing appraisal of dehydration tests with regard to their value in the study of frost hardiness is by no means exhaustive. The results indicate, however, that such tests yield comparative values of hardiness which are as dependable as those obtained from an actual freezing test. While dehydration tests should serve equally well for routine measurements of frost resistance in agricultural or horticultural practice, some modifications of the procedures, as they are described here, may be required before they will prove to be entirely suitable and preferable to actual freezing tests for this purpose. In any case, whether dehydration tests or freezing tests proper are used for determining frost hardiness, it appears that the accumulated evidence concerning the nature of the mechanism of frost injury, though available in papers and reviews (**4, 6, 7, 8**), has not been generally utilized in guiding the design of tests for this purpose. It is timely and useful therefore to present certain considerations based on this evidence and on the experimental results of the present paper, to serve as a guide in the laboratory testing of plants for frost hardiness.

It is essential that the facts concerning the mechanisms of freezing and freezing injury in plant tissues be recognized. There are two mechanisms by which plant tissues may freeze and correspondingly two modes of injury. Normally, when the temperature drop is slow, the process involves the for-

mation of ice crystals only in the intercellular spaces, or extracellularly as it will be referred to subsequently (**8**). These ice crystals grow as the temperature drops, at the expense of water derived through the dehydration of the cells adjoining these spaces. A progressive increase in the solute concentration of cellular fluids and a concomitant depression of the freezing point of the water contained therein parallels the drop in temperature and this, together with an action of the protoplasmic colloids in promoting undercooling (**11**), effectively prevents crystallization from occurring in the interior of the cells. If, however, the temperature drop occurs very rapidly in the tissue, then either because of an excessive supercooling of both the extracellular and intracellular fluids followed by extensive ice formation or because the rate of diffusion of water from the cells to externally located centers of ice formation cannot keep pace with the drop in temperature, intracellular freezing eventually occurs.

Intracellular freezing is invariably fatal to cells whether they are hardy or not because of a mechanical disorganization of the protoplasm caused by the ice crystals which form within them (**8**). On the other hand, where freezing is extracellular, the cell contents are merely concentrated by dehydration. Injury, if any, arises only from the effect which dehydration has on the protoplasm and protoplasmic membranes of the cells. Extracellular freezing, as has been emphasized by SCARTH (**6**) does not differ intrinsically from the process of dehydration by plasmolysis or desiccation although details of the mechanism of the volume contraction may vary from one process to another. In each instance the reduction in the protoplasmic and vacuolar volume increases with increasing dehydration. In plasmolysis this reduction in volume is achieved osmotically and is observed microscopically as a recession of the protoplast, independently, from the cell walls. Dehydration by desiccation or by freezing results from a diffusion or distillation of water through the cell membrane, and it is observed that the protoplast continues to adhere to the cell walls during the process. The walls, usually two opposite walls, invaginate and the cell as a whole collapses. It might be expected that these differences in mode of contraction would result in differences in degree of injury produced by plasmolysis on the one hand and desiccation or freezing on the other at equivalent levels of dehydration which may, indeed, be the case; but the injury associated with the contraction in each of these dehydration processes is largely a product of those effects which the removal of water from plant protoplasm has on its physical properties. With this removal of water from the protoplasm being the unique feature common to all these processes, the essential similarities between them, in respect to their injurious effects on cells, outweigh their differences. Thus a certain degree of sensitivity of the cells to injury by plasmolysis, for example, marks a closely corresponding although not necessarily identical degree of sensitivity of the cells to injury by desiccation or by freezing.

The testing for total frost hardiness in plant tissues must always involve

two independent procedures, first, a measurement of the relative facility with which intracellular ice formation can be induced in these tissues and, secondly, a measurement of the capacity of these tissues to withstand the sum of the various effects of dehydration and rehydration.

Consider first the testing for resistance to injury by intracellular freezing. Since intracellular freezing is always fatal to a cell in which it occurs, any resistance which a plant may develop or possess to injury of this type can only be such as to prevent its incidence. Because the mechanisms concerned in such case are still obscure, it would appear that this resistance can be effectively evaluated only by exposing the plant or plant tissue to environmental conditions favoring this type of freezing followed by an appraisal of the degree of its incidence. Such conditions could be achieved either by rapid freezing or by excessive supercooling in a freezing chamber followed by rapid ice formation. Rapid freezing is the more easily controlled and therefore the preferred of these methods. It is necessary to distinguish the effects due to intracellular freezing from those due to extracellular freezing and this can be achieved directly only through microscopic observation of the mode of freezing in the tissue slices mounted on a freezing stage. While the susceptibility of cells to intracellular freezing when exposed to a rapid drop in temperature in tissue sections might not be identical with that of cells of the intact plants, some correspondence in this respect would be expected. If intact plants are to be tested, direct observation of the freezing process is attainable only in those somewhat translucent plants where a considerable number of the individual cells can still be discerned within the intact structure of the plant when viewed under a low power objective. The behavior of cells undergoing intracellular freezing is characteristic and readily recognized under microscopic observation (**8**). In lieu of any such method of direct observation, some indirect criterion of the degree of incidence of intracellular freezing could be employed. In most instances the degree of incidence of intracellular freezing can be assessed by comparing the amount of injury obtained after slow freezing with that obtained after rapid freezing. Since cells are invariably killed at any freezing temperature if frozen intracellularly, the killing temperature with fast freezing will be far above that with slow freezing. It is necessary then, in comparing a number of different plants for relative susceptibility to intracellular freezing, to find, first, some freezing temperature which produces no injury to any of the plants when they are cooled slowly to this temperature and thawed at some standard rate. The same plants will then be cooled to this temperature at progressively more rapid rates and thawed at the standard rate until injury becomes apparent. Any differences between these plants with respect to injury will then be attributable to differences in the incidence of intracellular freezing. A standard period and temperature for recovery between freezings must be maintained. In addition to such rapid freezing tests, consideration of the mechanism of freezing indicates that the occurrence of one or the other type of freezing may be determined by observing

the rate at which the cell fluids can be concentrated by passage of water to externally growing ice crystals. Although other factors may also be involved, the permeability of the cell surface as well as the ratio of cell surface to cell volume will affect this rate of concentration. LEVITT and SCARTH (**2**) have shown that hardy cells are more permeable to water than non-hardy cells and that these hardy cells are less susceptible to intracellular freezing under conditions of rapid cooling. They recommend a plasmolytic determination of water permeability as a test for resistance to intracellular freezing. With the increase in ratio of surface to volume which accompanies reduction in cell dimensions, it is not surprising that the hardiest plants, namely those which are exposed to and tolerate rapid temperature fluctuations as well as extremes of low temperature, like trees and shrubs, have characteristically small cells. Intracellular freezing is probably of rare occurrence in these plants under any conditions of cooling. The considerable resistance of bacterial cells to injury by rapid freezing may be due to a particularly high order of the ratio of their surface to volume. On the other hand, herbaceous plants generally, and large-celled succulents in particular, are prone to intracellular freezing which must be a predominant factor in the injury of these plants by frost under natural conditions. Even hardened cabbage plants show intracellular ice formation under conditions which never produce intracellular ice formation in the cells of the bark of trees or shrubs (**8**). Cell size appears to be the one morphological or anatomical character which bears some consistent relationship to the total hardiness of plants. A preliminary appraisal of cell size, even without a rapid freezing test, may well serve as an index to that part of the hardiness of the plant which affords protection against injury by intracellular freezing.

Because of the predominant importance of the extracellular mode of freezing in perennial plants under normal conditions, testing for resistance to injury by this type of freezing assumes relatively much greater importance than testing for resistance to injury by intracellular freezing. The guiding principle in the design of tests for this type of hardiness is the essentially dehydrative character of extracellular freezing. This form of resistance is, therefore, amenable to testing in the laboratory at room temperature by means of the dehydration tests of plasmolysis and desiccation as well as by conventional freezing tests. Relative resistance to injury by dehydration can be measured in terms of the percentage survival of the cells of the plant tissue after being subjected to different freezing temperatures or, alternatively and much more quickly, after exposure to different degrees of dehydration in plasmolyzing solutions of varying concentration or against desiccating atmospheres of varying relative humidity. The linear correlations for such tests performed simultaneously on tissue samples taken from the bark of the black locust at various stages of hardening establishes the fact that the different tests are equally valid for estimating the relative hardiness of these tissues. It is indicated in these experiments, however, that equal dehydration intensities produced by these empirically chosen plas-

molysis, desiccation, and freezing tests are not strictly equivalent with respect to injurious effects on cells.

The lack of equivalence between the various tests stems partly from the peculiarities of the cell contractions associated with each of these dehydration processes and partly from the particular manner in which the dehydration tests have been carried out. The stresses and strains developed in the protoplasm in the contracting as well as in the expanding stages of the hydration changes to which they are subjected are somewhat different in each test even though the maximum degree of dehydration of the protoplast may be identical. The salt solutions employed in the plasmolysis tests are in themselves somewhat toxic, and the increase in injury with time observed in plasmolyzed cells exceeds the injury expected on the basis of dehydration alone. The most important lethal differences at comparable dehydration intensities in the tests, however, probably stem from differences in the rate and length of time of exposure to the dehydration conditions, in the rate of rehydration, and in the temperatures at which the tests are carried out. The effects of these and possibly other factors appear either to remain constant in a given test empirically devised or their contributions to the lethal action in the test bear a constant relationship to the dehydration intensity imposed. Thus the application of a given test to tissues of varying resistance to dehydration injury will yield results depicting a constant ratio between that resistance and the dehydration intensity applied. The ratio may vary for each empirical modification of procedure incorporated into the test, and it will be different from one general type of test to another. A plasmolysis, desiccation, or freezing test, run in a constant though empirical manner on a series of tissues of varying resistance to dehydration injury will, however, yield relative values of dehydration intensities which accurately reflect those capacities. The ratios of dehydration intensities required for equal degrees of killing will not be unity between the different tests but the ratios will parallel one another.

The choice of empirical procedure to be adopted with any of these types of tests will be based on the convenience with which the test can be performed on the tissue under observation, but a rigid adherence to the chosen procedure is essential for dependable comparisons. Actual calibration of the other dehydration tests against a freezing test is desirable. However, any actual freezing test which might be followed will itself be only relative with respect to any different freezing test and will not necessarily be any better correlated with actual conditions in the field (winter conditions) than are the dehydration tests which can be run at ordinary temperatures.

In the foregoing discussion the essential features which must be incorporated into tests of frost hardiness in plants have been described. The remaining discussion concerns the application of these tests to types of specific problems which are encountered in investigations of frost hardiness. Such problems can be classified broadly into two groups, one, the theoretical or fundamental studies where interest is attached to the mechanisms involved

in frost resistance, and the other, the practical studies which are concerned primarily with the testing of the capacity of plants to survive freezing conditions in the field.

The studies reported in the present series of papers, have, as their objective, the detection of relationships between changes in the chemical composition of the cellular components of the bark tissue of the locust tree and changes in its frost hardiness, and ultimately, the discernment of the chemical mechanism of frost hardiness. This is an example of the more theoretical type of investigation of frost hardiness. In such studies, it is necessary only that the direction of change in hardiness or the differences in hardiness be determined. The exact freezing temperature which the tissues can tolerate is of secondary importance. Any test, therefore, which will encompass the range of hardiness encountered in the black locust bark and which will also indicate the relative hardiness of these tissues is adequate. Furthermore since extracellular freezing is observed to be the most usual form of freezing in a small-celled tissue like the bark of trees, this form of freezing alone being capable of producing 100% killing at -5° C in mid-summer and on injury at all at -60° C in winter, the most important chemical changes involved in the total hardening mechanism would be those concerned with the development of resistance to extracellular freezing. It is not implied that resistance to intracellular freezing may not be produced in winter in the locust, rather it is emphasized that resistance to extracellular freezing constitutes the most important aspect of its total frost hardiness. In following the hardiness changes in the locust in relation to changes in chemical composition, only that part of the hardiness need be measured which consists of resistance to injurious effects of extracellular freezing or dehydration. It follows from previous considerations that plasmolysis or desiccation tests are not only eminently suitable but desirable for the type of investigations here pursued.

Another aspect of tests for hardiness for use in fundamental chemical investigations further differentiates them from practical tests. The survival capacity of the plant as a whole is not a prime consideration in such fundamental studies. Rather the survival capacity of a plant part or even of a group of cells, in relation to chemical composition is the point of emphasis. It becomes increasingly evident from studies of this kind that, ultimately, local variations in hardiness and chemical composition will have to be considered. Ideally the chemical composition of each cell or small group of cells should be studied in relation to the hardiness of the same cell or groups of cells. Plasmolysis or desiccation tests, requiring but minimal amounts of test material are especially suitable for such studies.

Where, on the other hand, the objective of hardiness testing is a prediction of the capacity of a plant to survive when exposed to freezing conditions in the field, some modification of the present dehydration tests or the use of actual freezing tests alone may be required. In the first place a measure of the hardiness of every part of the plant is generally necessary. If plas-

molysis or desiccation tests are to be applied, tissue sections for the tests should be taken from all parts of the plant. Injury to critical tissues like the primary or secondary meristem must certainly be assessed because this injury, however localized or restricted, could eventually lead to altered growth or even death of the plant, as is well recognized in horticultural practice. It is important, also, that resistance to injury by intracellular freezing must be determined. The possible incidence of injury from intracellular ice formation can never be ignored in the case of herbaceous plants. Actual freezing tests must be employed for this purpose. An appraisal of the relative contributions of both types of freezing to the total injury under different freezing conditions might well be a guide to the climatic conditions most suitable for cultivation of the plant under consideration. Although the resistance to intracellular freezing must always be taken into account, the measurement of resistance to injury produced in extracellular freezing is still the most important aspect of the practical testing for frost resistance.

Dehydration tests can prove most useful in this regard. An important advantage of the plasmolysis or desiccation tests derives from the fact that there is never any question but that resistance to dehydration, and therefore to extracellular freezing, is being measured by these methods. Because of the small amounts of tissue required in these tests compared to those required for a satisfactory freezing test, the course of hardiness development in one or many plants which are being subjected, as in agronomic practice, to varying environmental conditions over an extended period of time can be followed continuously and conveniently through their use without serious detriment to the plant or plants. For these reasons the plasmolysis and desiccation tests where logically applied will afford methods for the practical testing, simultaneously and with limited space and equipment, of the hardiness of a very large number of plant individuals.

The results of the dehydration tests reported in this paper also indicate that plants which are frost resistant are also drought resistant. Dehydration tests can be used equally well for the determination of the capacity of plants to resist drought injury, and their use for this purpose should aid in the selection of plants possessing combined resistance to drought injury and to injury by extracellular freezing.

Summary

The validity of the use of plasmolysis and desiccation tests for estimating the resistance of the bark tissue of the black locust to injury by extracellular freezing has been examined and established by comparing such tests with actual freezing tests performed under natural and standardized laboratory conditions.

Considerations based on published evidence concerning the mechanism of freezing and freezing injury in plants and on the results of the correlation studies described, are presented in a discussion to serve as a guide to the testing of frost hardiness of plants in the laboratory in general. Special

emphasis is accorded to the usefulness and the advantage of dehydration tests for this purpose.

The authors wish to thank the Herman Frasch Foundation for Chemical Research for a grant of funds in support of a five-year program of research into the problem of winter hardiness in plants. The present paper constitutes a report on a part of this research.

LITERATURE CITED

1. Briggs, D. R. and Siminovitch, D. The chemistry of the living bark of the black locust tree in relation to frost hardiness. II. Seasonal variations in the electrophoresis patterns of the water-soluble proteins of the bark. Archiv. Biochem. **23**: 18–28. 1949.
2. Levitt, J. and Scarth, G. W. Frost-hardening studies with living cells. II. Permeability in relation to frost resistance and the seasonal cycle. Canadian Jour. Res. C. **14**: 285–305. 1936.
3. Levitt, J. and Siminovitch, D. The relation between frost resistance and the physical state of protoplasm. I. The protoplasm as a whole. Canadian Jour. Res. C. **18**: 550–561. 1940.
4. Levitt, J. Frost Killing and Hardiness of Plants. A critical review. Burgess Publishing Co., Minneapolis. 1941.
5. Scarth, G. W. and Levitt, J. The frost-hardening mechanism of plant cells. Plant Physiol. **12**: 50–78. 1937.
6. Scarth, G. W. Dehydration injury and resistance. Plant Physiol. **16**: 171–179. 1941.
7. Scarth, G. W. Cell physiological studies of frost resistance. New Phytologist **43**: 1–12. 1944.
8. Siminovitch, D. and Scarth, G. W. A study of the mechanism of frost injury to plants. Canadian Jour. Res. C. **16**: 467–481. 1938.
9. Siminovitch, D. Reported at the Annual Meeting of the Amer. Soc. Plant Physiol., Philadelphia, Pennsylvania. 1940–1941.
10. Siminovitch, D. and Levitt, J. The relation between frost resistance and the physical state of protoplasm. II. The protoplasmic surface. Canadian Jour. Res. C. **19**: 9–20. 1941.
11. Siminovitch, D. and Briggs, D. R. The chemistry of the living bark of the black locust tree in relation to frost hardiness. I. Seasonal variations in protein content. Archiv. Biochem. **23**: 8–17. 1949.

THE SPROUTING OF SWEETGUM IN RELATION TO SEASON OF CUTTING AND CARBOHYDRATE CONTENT

KARL F. WENGER

SOUTHEASTERN FOREST EXPERIMENT STATION, FRANKLIN, VIRGINIA

Received December 7, 1951

Because vegetative succession in the range of loblolly pine is toward a hardwood climax, successful pine regeneration sooner or later requires reduction of hardwood competition. It would be a great deal easier to cope with the competing hardwoods if they could not sprout. All hardwood species in this type are capable of sprouting, however, although there are differences among species. To devise effective and efficient methods for controlling hardwoods, the forester must know how and why environmental conditions and characteristics of the parent stem affect sprouting.

In the southern Atlantic coastal plain, sweetgum (*Liquidambar Styraciflua* L.) is a major component of the hardwood understory. Its prevalence and its ability to sprout quickly, vigorously, and persistently make it one of the most serious competitors of pine seedlings.

Reports concerning other species indicate that site quality, size of the parent tree and season of cutting are the most important factors influencing the sprout growth of a species. Good sites result in more vigorous sprouting than poor sites (**25, 29**). Sprouting generally seems to increase with the diameter (at breast height) of the parent tree up to five inches and decreases with diameters above six inches (**20, 22**). Sprouting is least from stumps cut during the period of active growth and greatest from those cut during the dormant season (**6, 25**). Because of year-to-year variation in seasons, STOECKLER (**25**) related sprouting to phenological development and found that cutting when the leaves were nearly full size produced the least vigorous sprouts.

The seasonal trend of sprouting vigor is believed to be the result of the seasonal fluctuation of carbohydrate reserves (**1, 25**). Total carbohydrate reserves fluctuate in about the same pattern as sprouting vigor, declining sharply during spring growth to a minimum in early summer, building up to a peak in fall and going through a slight and gradual decline during the winter. This trend has been found in nearly every species studied, including fruit trees, forest trees, and woody shrubs (**12, 14, 18, 24**). ALDOUS (**1**) reported the absence of a seasonal trend in the carbohydrate content of buckbrush, but this finding is questionable in view of the consistency with which such a trend has been found in other species.

Presumably a plant will die if food reserves drop below some critical level. In Connecticut annual mowing of brush-invaded pastures for four years at different times during the year resulted in nearly complete disappearance of woody growth from plots cut in June, July, or August while

plots cut in winter, April or May showed little change (**5**). Perhaps the treatment could be made more effective in the southern coastal plain where the longer growing season might permit more than one annual cutting.

Although the effects of site, season of cutting, and size of the parent tree probably hold in a general way for sweetgum in the southern coastal plain, the longer growing season might extend or shift the period of minimum sprouting. The presence of the overstory also might influence sprouting behavior. Accordingly, a study was begun in the spring of 1947 at the Santee Experimental Forest located in the Francis Marion National Forest 30 miles north of Charleston, South Carolina, to answer the following questions: (a) At what stage of plant development is sprouting least vigorous? (b) Does the destruction of successive generations of sprouts during a single season reduce sprouting vigor? (c) Is sprouting vigor related to the size of the parent stem? (d) How is sprouting vigor related to carbohydrate reserves? The effects of site quality on sprouting are equally important but the means available were not adequate for study of that factor.

Experimental procedure

An aggressive understory of nearly pure sweetgum in an old-field stand of loblolly pine on a moderately good site was chosen for the study. The size of trees used was restricted to less than three inches in diameter at breast height. Choice of trees was confined to those of seedling origin whenever possible.

Season of cutting

For the determination of the period of minimum sprouting, 10 trees were cut every two weeks throughout the growing season, from April 28 to September 15, 1947. At the time of cutting, the stage of development of each parent tree was recorded in terms of size and texture of leaves, terminal shoot growth, terminal bud formation, and the progress of shoot hardening. One year after the parent stem had been cut the sprouts were counted, weighed, and measured.

The time of cutting the parent tree had no effect on the total height, total fresh weight, or number of sprouts produced per stump during the year after cutting. The relationships between the diameter of the parent tree at breast height (d.b.h.) and these sprout attributes were highly significant, however, and are expressed by the following equations: Total height in feet = 2.0 + 14.0 (d.b.h.); Total weight in grams = −51.5 + 291.8 (d.b.h.); Number of sprouts = 4.3 + 6.6 (d.b.h.).

Much of the variation in total height and weight was the result of differences in numbers of sprouts, which were not related to time of cutting. Variation from this source is eliminated if the height and weight of the average sprout from each stump are used. A regression analysis showed that both the height and weight attained by the average sprout in the year following cutting depended on the date of cutting (stage of phenological devel-

opment) of the parent tree. Average height and weight were also related to the diameter of the parent tree. Figure 1 shows the effect of parent-tree diameter on average height of one-year-old sprouts and the trend of sprout height in relation to phenological development of the parent tree at the time of cutting. Stumps cut early in May, when the leaves that had emerged first were nearly full size, produced the shortest sprouts. This result agrees with those of other studies, but the second minimum, in August, has not been reported before. Fitting the curve by the method of least squares showed that this late summer minimum is a significant variation in the trend. The calculated curve limits the duration of the minimum period to about two weeks, although the data in figure 1 show that it actually lasted

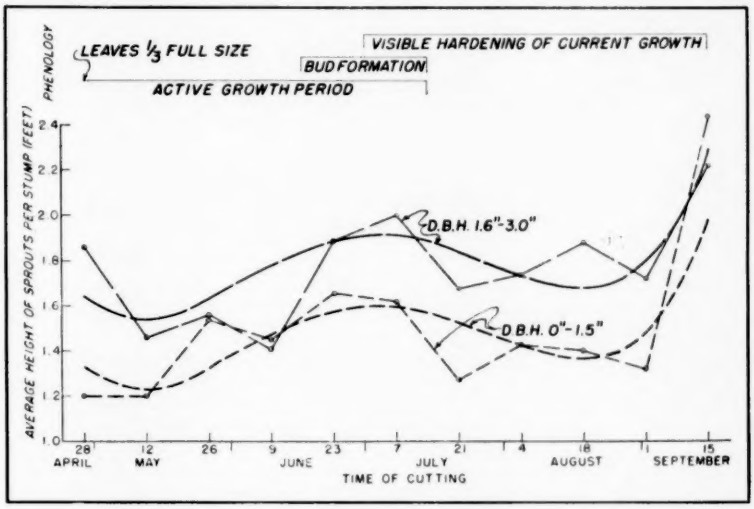

FIG. 1. Average height of one-year-old sprouts in relation to diameter and time of cutting of parent tree.

nearly six weeks for the year in question, beginning shortly after terminal buds were formed. Mortality of stumps was negligible.

CUTTING SUCCESSIVE GENERATIONS OF SPROUTS

To study the effect of cutting successive generations of sprouts from the same stump, two cutting treatments were replicated six times within two diameter classes (0.0 to 1.5 inches, and 1.6 to 2.0 inches) on three initial cutting dates. Initial cutting dates were four weeks apart beginning April 28. On each initial cutting date 24 trees (12 in each diameter class) were cut; eight weeks later the sprouts were cut; after a second eight-week period the sprouts on 12 stumps (six in each diameter class) were cut again. The trees were chosen at the start, and treatments were randomly assigned. The interval of eight weeks between cuttings on the same stump was chosen after

observing the growth of sprouts from the first cuttings on April 28. One year from the date of final cutting for each stump the sprouts were counted, weighed, and measured.

The dates of the final cuttings coincided with those for the season-of-cutting experiment, so that one-year-old second and third generation sprouts could be compared directly with one-year-old first generation sprouts. Third generation sprouts from stumps cut initially in May and June made most of their growth the following year; in fact, most third generation sprouts from the June cutting did not arise till the next spring. The mean values for the different levels of the three main classifications are presented in table I with significant differences marked by asterisks. As in the season-of-cutting experiment, stumps of larger trees produced both larger and more numerous sprouts.

TABLE I

SPROUT SIZES AND CARBOHYDRATE CONTENT ONE YEAR AFTER FINAL CUTTING BY FACTOR LEVELS (SUCCESSIVE CUTTINGS).

Factor levels		Sprouts per stump					Carbohydrate content	
		Number	Total height	Total weight	Average height	Average weight	Stump	Root
			ft.	*gm.*	*ft.*	*gm.*	*mg./gm.*	*mg./gm.*
Diameter of parent tree (inches)	0.0-1.5	8.6*	11.71*	162*	1.40*	19.8*	33.6	76.0*
	1.6-3.0	15.2*	26.11*	418*	1.72*	28.8*	28.8	44.8*
Number of sprout cuttings	0	13.0	20.95	326	1.62	23.6	27.6	56.7
	1	12.2	19.69	333	1.63	28.6*	30.0	57.1
	2	10.6	16.09	210	1.44	20.6*	33.6	67.3
Time of initial cutting	April 28	10.6	17.61	345	1.69	33.6**	24.6*	46.1*
	May 26	11.8	18.60	253	1.47	18.7*	30.0	56.7
	June 23	13.3	20.53	271	1.52	20.5*	36.6*	78.3*

*Vertically aligned asterisks indicate significant or highly significant differences.

Whether the number of sprout generations previously destroyed affected the number and size of sprouts in the final crop is somewhat uncertain. The one significant difference, that difference between the average weights of second and third generation sprouts, can be traced directly to exceptionally heavy second generation sprouts from a single large stump of the series started on April 28. The number, total height, average height, and total weight of third generation sprouts also were less than those of second generation sprouts, however, although the differences were too small to be significant. In view of the results achieved by BROWN (5) with annual mowing of brush-invaded pastures, it is possible that there was a real trend toward lower vigor in successive generations of sprouts from the same stump.

The effect of time of initial cutting seems to have been expressed in the form, rather than in the number or height, of sprouts. Although the excep-

tionally high value mentioned above inflated the average weights of sprouts from the April 28 cuttings, the other sets begun on April 28 (all first and third generation sprouts and second generation sprouts from smaller stumps) were also heavier. Consequently, there is no reason to doubt that sprouts from cuttings started on April 28 were significantly heavier than sprouts from cuttings started later. Since the trend in height of these same sprouts was neither significant nor well-defined, the differences in weight suggest that the sprouts from the May 26 to June 23 cuttings were more slender than those from the cuttings started on April 28.

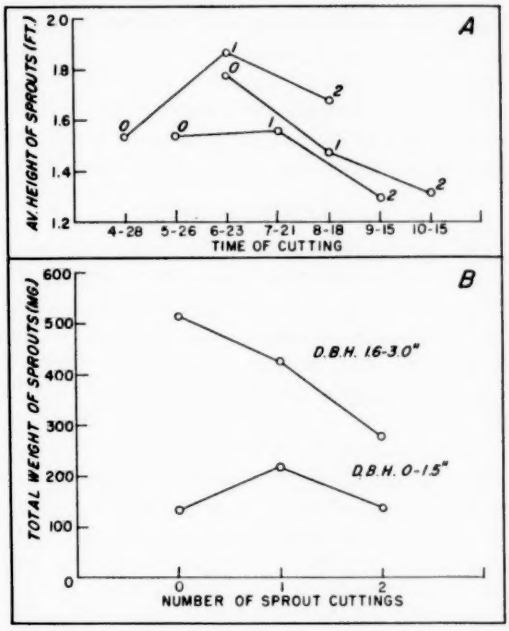

FIG. 2. The effect of number of cuttings, time of cutting, and parent-tree diameter on average height and total weight of one-year-old sprouts.

The interaction of the number of cuttings and time of initial cutting on the number of sprouts was also significant. In the series started on May 26, the number of sprouts increased with each additional cutting while in the series started on April 28 and June 23 the number of sprouts tended to decrease. There seems to be no reason why the number of sprouts should vary in that manner.

Figure 2 A shows the trends in average height on the same basis, although differences among them did not quite reach the 5% level of significance. Comparison of figure 2 A with figure 1 suggests that the number of sprout generations previously destroyed reduced sprouting vigor when cutting was started near the end of the period of active terminal growth or later but not

when it was started before that time. Second and third generation sprouts (fig. 2 A) from cuttings started on April 28 were as tall as first generation sprouts (fig. 1) but third generation sprouts from cuttings started after April 28 were shorter than first generation sprouts. They were as tall as first generation sprouts from cuttings made at the time of minimum sprouting vigor, however (fig. 1). The comparison between figure 2 A and figure 1 also suggests that the variation among the trends in average height (interaction of number of cuttings and time of initial cutting on average height of one-year-old sprouts) is of greater significance than the statistical tests indicate.

The interaction of parent-tree diameter and number of sprout cuttings on total weight of sprouts per stump was also significant and is shown graphically in figure 2 A. Total weight of sprouts from parent trees of larger diameter followed a definite downward trend with number of cuttings while sprouts from smaller parent trees followed no well-defined trend. Mortality among stumps of the successive cuttings was negligible.

Carbohydrate content

When the sprouts from treated trees were harvested one year after the final cutting, samples of stumps and roots were taken to determine the carbohydrate content after sprouting. When the sprouts from the seasonal cuttings, for which 10 trees were cut every two weeks during the growing season of 1947, were harvested in 1948, five of the 10 stumps were randomly selected and two samples were taken from each, one close to the point of sprout origin and one from the root close to but distinctly below the root collar. Samples were taken in the same way in 1948, one year after the date of final cutting, from all stumps and roots of the successive cuttings, for which three sets of 24 trees each had been treated during three periods beginning four weeks apart in 1947.

During the same year, 1948, a series of stump and root samples for carbohydrate determinations were taken from uncut trees to furnish control data. The original plan did not provide for a study of food reserves and no samples for carbohydrate determination were collected from check trees in 1947, when the initial cuttings were made. Collection of check samples the following year when the one-year-old sprouts from treated stumps were harvested was believed to be satisfactory, however, for, while the level of carbohydrate concentration may vary from year to year, its seasonal trend, judging from other studies, remains relatively constant. As quickly as possible after collection, usually within two hours, the samples were placed in an oven at 70° C, dried to constant weight, and stored to await carbohydrate analysis. The samples were ground in a Wiley mill to pass a 20-mesh sieve. One-gram portions were used in the analysis.

Carbohydrates were extracted by a modification of the enzyme digestion method described by Hepting (11). The essential steps in that method are (a) the extraction of resins and oils with ether, (b) the digestion of starch to sugars by Takadiastase, (c) the removal of tannins and gums by lead

acetate clearing, and (d) the acid hydrolysis of all sugars to glucose. A series of trials showed that the ether extraction of resins and oils was not necessary with sweetgum. Glucose was determined by the Shaffer-Somogyi method as described by HEINZE and MURNEEK (**10**).

The total, or absolute, quantity of carbohydrates as well as the concentration must be determined to locate the principal storage tissue. In addition, the weights of bark and wood must be in the same ratio as they are in the intact tree. Samples collected throughout the growing season accordingly were treated, before grinding, as follows: First they were trimmed to symmetrical sectors of the stem cross-section (the whole cross-section of smaller stems was used); then they were weighed; the bark was removed and saved; and finally the wood was weighed again. One-gram samples of wood and bark from both stems and roots were analyzed. The results are presented in table II. The separation by diameter classes was made because the ratio of bark to wood decreases with increasing diameter. The concentrations were as follows: Stem wood, 0.0285 gm. sugar/gm.; Stem bark, 0.0694 gm. sugar/gm.; Root wood, 0.1170 gm. sugar/gm.; Root bark, 0.0787 gm. sugar/gm. The small difference between stem bark and root bark was not significant, but all other differences were highly significant.

TABLE II

PROPORTION OF TOTAL CARBOHYDRATE RESERVES OF STEM AND ROOT FOUND IN WOOD AND BARK OF SWEETGUM.

Parent-tree diameter	Stems		Roots	
	Wood	Bark	Wood	Bark
In.	%	%	%	%
1	74.4	25.6	91.6	8.4
2	79.0	21.0	93.1	6.9
3	81.0	19.0	91.4	8.6

Although it was impractical to obtain stump and root weights to compare the total amounts of carbohydrates, it can be stated that the roots weighed more than the stumps in all cases. The root wood, therefore, contained by far the greatest amount of carbohydrate reserves.

In one study of sugar maple, with larger trees than those in this study, it was found that outer stem wood contained more carbohydrates than inner wood (**14**). Accordingly, some of the sweetgum samples were selected and divided into inner and outer portions, the outer portion including the growth of about two years. The differences in carbohydrate concentration were small and not significant. Apparently these trees were so young that the whole stem and root were still involved in transformation and translocation of carbohydrates in contrast to the larger sugar maple trees in which the differences probably were related to the inactivation of older stem wood.

Since the bulk of reserve carbohydrates are located in the wood (table II), the bark was not analyzed in the seasonal and successive cuttings or in the uncut trees. Figure 3 shows the seasonal trends of carbohydrate concen-

trations in the roots and stems of uncut trees and of the seasonal cuttings after sprouting. The trend for roots of uncut trees only was significant. There was no difference in carbohydrate concentration in roots or stems between trees of different diameters in either the uncut or the treated sets. Larger trees, then, contained the greater absolute quantity of carbohydrates.

The difference between the trends for uncut and treated trees was caused by sprout growth since the values for the uncut trees are, in fact, an estimate of what the treated trees would have had if they had not been cut. Root growth probably required the same amounts of carbohydrates in both cases unless some roots of the treated trees died after cutting. In that case the differences in figure 3 are less than the amounts of carbohydrates actually used by sprout growth.

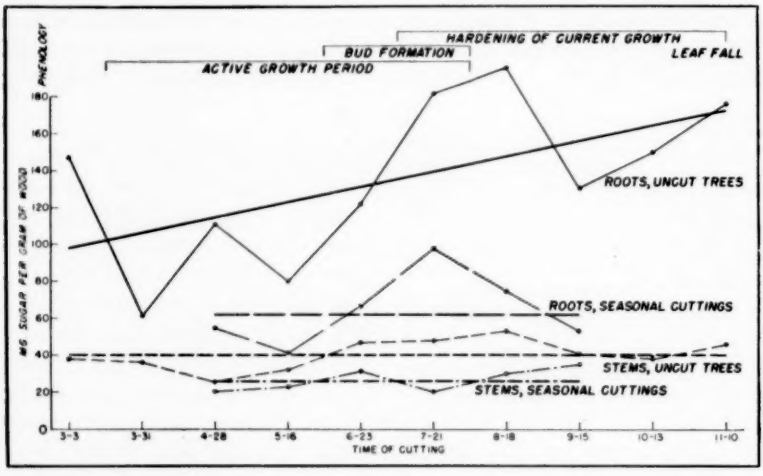

FIG. 3. Carbohydrate content of roots and stems of sprouted trees one year after cutting and of uncut trees.

Carbohydrate contents of stumps and roots of the successive cuttings are shown in table I. Roots of smaller trees had a significantly higher concentration after sprouting than those of larger trees. Carbohydrate concentrations in both stumps and roots were significantly higher for later initial cutting dates.

The interaction of number of cuttings and time of initial cutting on the carbohydrate concentration in the roots is shown in figure 4. The interaction was statistically significant, but the data showed that the difference responsible for significance should be attributed to chance rather than to treatment. The high value for single cuttings (0) made on June 23 was caused by an excessively high carbohydrate concentration in only one root. The main effect, number of cuttings (see table I), might have been significant if the value in question had been of the same order of magnitude as the two earlier single cuttings.

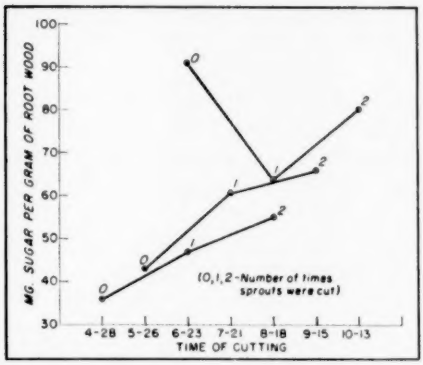

FIG. 4. The effect of number of cuttings and time of cutting on carbohydrate content of roots one year after final cutting.

Discussion

If the vigor of sprouting, expressed by the size attained by sprouts in one year, depended on the amount of reserve carbohydrates, larger stumps should produce larger sprouts because of the greater amount of reserve carbohydrates. This expectation implies a level of carbohydrate concentration that is critical for sprout growth. Considering also that the greater amount of foliage of the larger sprout clumps should produce a greater amount of carbohydrates, the carbohydrate concentration in the roots after growth of sprouts should be at least as high in the larger as in the smaller stumps. With respect to the change in size of sprouts in relation to the time of cutting, one would expect sprouts to increase constantly in size during the period of accumulation because carbohydrate concentration increases constantly during that period. These expectations apparently are realized with respect to diameter in the experiment on the season of cutting (figs. 1 and 3). In the successive cuttings, however, where the effect of diameter was determined more precisely, larger stumps contained a lower residual carbohy-

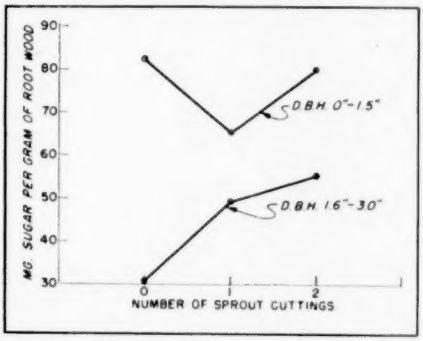

FIG. 5. The effect of number of cuttings and parent-tree diameter on carbohydrate content of roots one year after final cutting.

drate concentration than smaller stumps but produced larger and more numerous sprouts (table I). Seasonally, sprout growth and carbohydrate concentration were not well correlated: carbohydrates increased constantly during the accumulation period while sprout growth fell to a second minimum. The inverse relationship between sprout growth and carbohydrate content is shown in striking fashion upon comparing the effect of number of sprout cuttings on the total weight of sprouts from stumps of different sizes (fig. 2 B) with the carbohydrate content of the roots after sprouting (fig. 5) and is also evident upon comparing the trends of average sprout heights in figure 2 A with their root carbohydrate content after sprouting in figure 4.

It is clear, therefore, that under the conditions of these experiments, carbohydrates did not control the growth of sprouts but the amount of sprout growth determined the amount of carbohydrates used. Data recorded by several other workers support this conclusion; for example, FINCH (**8**) found that in regularly bearing apple varieties terminal growth was greater and carbohydrate content was less than in biennially bearing varieties in the off year while shyly bearing varieties were intermediate in both respects. In fruit trees (**12, 26**) the seasonal behavior of carbohydrates indicates that they are used in whatever amounts needed and the soluble products move whatever distance is required to reach the growing regions. There seems to be no obstacle to exhaustion of reserve carbohydrates throughout the plant to satisfy growth requirements, yet it has been demonstrated that food reserves within a short distance of the growing points usually suffice (**7**). Apparently under normal conditions, lack of carbohydrates usually is not the limiting factor in growth, but even under the most drastic treatments imposed in this study carbohydrates were not the limiting factor in the size sprouts attained. Had the treatments been continued in following years, carbohydrates might have become limiting.

Nitrogen, mineral nutrients and moisture supply are all, at times, critical in the growth of a plant. Certain facts indicate, however, that they, also, are not important in determining the seasonal trend of sprouting. Several facts about nitrogen metabolism should be noted in this respect: the carbohydrate–nitrogen ratio is more closely related to growth than nitrogen alone (**17**); meristematic regions maintain a higher nitrogen level than others (**15**); and the end of active growth is regarded as the result of carbohydrate accumulation (**28**), that is, the increase in the C/N ratio. But sprouts grow during the same season from the stumps cut after terminal growth of the parent trees has stopped, yet at this time the C/N ratio is higher in the stump than in the apical meristems. Evidently something other than carbohydrates or nitrogen determines the seasonal trend of sprouting vigor.

Variations in mineral nutrients or moisture supply also fail to provide a satisfactory explanation. The seasonal trend observed in this study is essentially the same as that recorded for many other species in many different places under what must have been greatly different soil moisture and fertility conditions.

A better explanation may be that the mechanism of apical dominance also controls the seasonal trend of sprouting vigor. Auxin inhibits the sprouting of lateral buds (**23**). It is produced by growing buds and young leaves and moves down the stem, where cambial activity follows its progress (**3, 4**). Its production in terminal buds follows a seasonal trend from an indetectible amount during the dormant season to a peak just before the most rapid growth in length, appearing first as the terminal buds begin to swell in the spring (**3**). Conceivably, then, the higher the auxin concentration in the stump of a tree at the time of cutting, the less vigorous the sprouts.

Because little is known about the relation of auxin to dormant buds, several assumptions must be made in accepting this explanation. In the portion of the tree stem where dormant buds are effectively suppressed, the auxin concentration must remain above the critical inhibitory level throughout the period of favorable growing conditions; the susceptibility of dormant buds to auxin inhibition must vary, otherwise all sprouts from cuttings made during the growing season would arise under the same degree of inhibition and would all be the same size; and the degree of inhibition under which sprouts arise must persist and affect their growth for at least a year. THIMANN (**27**) suggested that roots, stems, and buds all react similarly to auxin but at different concentrations, the optimum for stems being much higher than for buds; consequently it is possible that there might be seasonal differences in response as well. One case of apparent persistence of a hormone effect has been reported: APPLEMAN (**2**) found the vigor and yield of potato plants related to the size of the seed piece and attributed it to a growth hormone produced in an amount proportional to the size of the seed piece.

The second minimum of sprouting vigor may be related to changing day length. The phenological development of many southeastern forest tree species seems to be partly controlled by photoperiod (**13, 16**). If cessation of growth in length and terminal bud formation is brought about by shortening of the day, the sprouting of dormant buds would seem to be effectively checked even if auxin concentration dropped below the critical level. Upon cutting, the dormant buds in the stump would immediately gain the benefit of all of the disproportionately large root system and, since response to photoperiod can be modified by nitrogen supply (**21**), might be able to sprout and grow. The influence of suddenly increased nutrients would become progressively less until no longer effective as the days shortened. In this study most of the sprouts from stumps cut after the middle of August did not grow till the following season. This explanation requires the assumption that the inhibition exerted by shortened photoperiod also persists for at least a year.

While the proposed explanation is admittedly highly speculative, it is nevertheless probable that sprouting vigor is controlled by a hormone mechanism. STOECKLER (**25**) made double cuttings of aspen, first at a height of 36 inches and then at 12 inches after 3 to 24 hours. Stumps cut once pro-

duced many more sprouts than twice-cut stumps. Double cuttings with a three-hour interval produced fewer sprouts than those with longer intervals. These time intervals hardly permit the operation of anything but a hormone, or hormones. The results suggest positive, stimulating action rather than a negative one but it is quite likely that two or more hormones of different action affect sprouting vigor. If apical dominance is indeed a hormone effect, the successive cuttings in this study furnish another suggestion that hormone action is involved in sprouting vigor. Stumps cut once produced a total of 13 sprouts per stump; when sprouts were cut back once, a total of 22 sprouts was produced; when sprouts were cut back twice, a total of 30 sprouts was produced. Apparently buds that sprouted first immediately suppressed the remaining ones.

The positive relation between diameter of the stump and number of sprouts results from the greater opportunity for the formation of secondary dormant buds with larger diameter. Sweetgum does not seem to produce adventitious buds readily. Among all the stumps in this and another related study only one produced sprouts from what were unmistakably adventitious buds. There are not enough dormant buds formed during normal growth to account for the number of sprouts produced, but secondary dormant buds grow from bud initials in the axils of the scales of the parent bud (**9, 19**). Sprouts were frequently observed arising in horizontal rows in precisely the way HAHNE (**9**) found secondary dormant buds arranged in pear. This lateral arrangement also reduces the tendency for buds that sprout first to inhibit others in the cluster, and a larger stump tends to produce more sprouts because a greater percentage of buds are laterally arranged.

The effect of parent-tree diameter on the size of sprouts is, most likely, the result of increased moisture and nutrients. Extension of lateral roots is more or less proportional to stem diameter, and the roots, spreading in all directions, tend to occupy a circular area. The roots of a two-inch tree, then, occupy not twice as much area, but more nearly four times as much as the roots of a one-inch tree, the area occupied increasing as the square of the relative increase in diameter rather than as a linear function of it. Sprouts from larger stumps therefore have relatively more moisture and nutrients available than those from smaller stumps and thus grow larger. This explanation is not in contradiction to the preceding discussion of possible hormone control of sprouting vigor. The seasonal trend, controlled by the postulated hormone mechanism, holds for all diameters, but at a higher level for larger diameters because of the greater supply of moisture and nutrients. The same holds true for the effect of site quality reported by other workers. Differences in sprouting vigor between sites are probably caused largely by moisture and nutrient differences but seasonal variations on a given site are attributable to the hormone system.

Summary

A study of sweetgum was made to determine (a) the time of least vigorous sprouting during the growing season in terms of phenological develop-

ment, (b) the sprouting vigor in relation to the size of the parent tree, (c) the effect on sprouting vigor of the destruction of successive generations of sprouts during the same growing season, and (d) the sprouting vigor in relation to carbohydrate reserves.

The seasonal trend of sprouting vigor (size attained by one-year-old sprouts) was studied by cutting trees at two-week intervals during the growing season. Another group was treated in such a way that first, second, and third generation sprouts could be compared at different times during the growing season. Observations of number, height, and fresh weight of sprouts were made one year after final cutting in all treatments. Diameter at breast height of all trees was recorded before cutting. When final observations were made, stump and root wood samples were collected from all treated trees and from uncut trees for carbohydrate determinations.

Sprouting vigor, expressed as the height or weight of one-year-old sprouts, followed a pronounced trend by date of cutting with two minima, the first when leaves that had emerged earliest reached full size and the second during the late summer hardening period. Sprout size was directly related to parent-tree diameter, large trees (1.5 to 3.0 inches) producing larger sprouts than small trees (0.0 to 1.5 inches).

The number of sprouts did not follow a seasonal trend but was dependent on diameter of stumps (parent-tree diameter), large stumps producing more sprouts than small stumps.

The destruction of successive generations of sprouts from the same stump during the same growing season apparently caused a decline in sprout vigor. Second and third generation sprouts from final cuttings made before September 15 were as tall as first generation sprouts, but third generation sprouts from final cuttings made on or after September 15 were much shorter than first generation sprouts. Sprouts from cuttings started on May 26 and June 23 were lighter in weight than those from cuttings started on April 26 but were not significantly shorter, suggesting that they were more slender. Sprouts from larger stumps decreased in total weight with number of generations previously destroyed while those from smaller stumps did not.

The study of carbohydrate reserves showed that, under the conditions of these experiments, the size and number of sprouts was not dependent on carbohydrate content.

The possible roles of nitrogen, other mineral nutrients, moisture, and hormones in determining the seasonal trend were discussed. It seems most likely that a hormone system related to that controlling apical dominance is the chief factor governing the seasonal trend of sprouting vigor.

The study was a cooperative project of the United States Department of Agriculture, U. S. Forest Service, and Duke University, Graduate School of Arts and Sciences. The assistance and support rendered by the following individuals is gratefully acknowledged: Dr. I. T. Haig and Dr. G. M. Jemison, while they were, respectively, Director, and Chief of the Division of Forest Management of the Southeastern Forest Experiment Station; Dr.

P. J. Kramer, Botany Department, Duke University; Dr. C. F. Korstian, Dean of the School of Forestry, Duke University; and the staff of the Central Coastal Plain Branch of the Experiment Station, Charleston, South Carolina.

LITERATURE CITED

1. Aldous, A. E. The eradication of brush and weeds from pasture lands. Jour. Amer. Soc. Agron. **21**: 660–666. 1929.
2. Appleman, C. O. Special growth-promoting substances and correlation. Science **48**: 319–320. 1918.
3. Avery, G. S., Jr., Burkholder, P. R., and Creighton, H. B. Production and distribution of growth hormone in shoots of Aesculus and Malus, and its probable role in stimulating cambial activity. Amer. Jour. Bot. **24**: 51–58. 1937.
4. Boysen-Jensen, P. Growth Hormone in Plants. Translated and revised by G. S. Avery, Jr. and P. R. Burkholder. McGraw-Hill Book Co., New York and London. 1936.
5. Brown, B. A. Effect of time of cutting on the elimination of bushes in pastures. Jour. Amer. Soc. Agron. **22**: 603–605. 1930.
6. Buell, J. H. Effect of season of cutting on sprouting of dogwood. Jour. Forestry **38**: 649–650. 1940.
7. Curtis, O. F. The upward translocation of foods in woody plants. II. Is there normally an upward transfer of storage foods from the roots or trunk to the growing shoots? Amer. Jour. Bot. **7**: 286–295. 1920.
8. Finch, A. H. Physiology of apple varieties. Plant Physiol. **10**: 49–72. 1935.
9. Hahne, B. The origin of secondary dormant buds in deciduous fruit trees. Univ. California Publ. in Bot. **13**(5): 125–127. 1926.
10. Heinze, P. H. and Murneek, A. E. Comparative accuracy and efficiency in determinations of carbohydrates in plant material. Missouri Agr. Exp. Sta. Res. Bull. 314. 1940.
11. Hepting, G. R. Reserve food storage in shortleaf pine. Phytopathology **35**: 106–119. 1945.
12. Ishibe, O. The seasonal changes in starch and fat reserves of some woody plants. Memoirs College of Sci. Kyoto Imp. U. Series B 11(1). 1935.
13. Jester, J. R. and Kramer, P. J. The effect of length of day on height growth of certain forest tree seedlings. Jour. Forestry **37**: 796–803. 1939.
14. Jones, C. H. and Bradlee, J. L. The carbohydrate content of the maple tree. Vermont Agr. Exp. Sta. Bull. 358. 1933.
15. Karmarker, D. V. The seasonal cycles of nitrogenous and carbohydrate materials in fruit trees. Jour. Pom. and Hort. Sci. **12**: 177–221. 1934.

16. KRAMER, P. J. Effect of variation in length of day on growth and dormancy of trees. Plant Physiol. **11**: 127–137. 1936.
17. KRAUS, E. J. and KRAYBILL, H. R. Vegetation and reproduction with special reference to the tomato. Oregon Agr. Exp. Sta. Bull. **149**. 1918.
18. LECLERC DU SABLON, M. Recherches physiologiques sur les matieres de reserves des arbes. Rev. Gen. Bot. **16**: 341–368; **18**: 2–25, 82–96. 1904; 1906.
19. LIMING, F. G. Origin and growth of dormant buds in oaks. Jour. Forestry **38**: 226. 1940.
20. LITTLE, S., JR. Relationships between vigor of resprouting and intensity of cutting in coppice stands. Jour. Forestry **36**: 1216–1223. 1938.
21. ROBERTS, R. J. and STRUCKMEYER, B. E. The effects of temperature and other environmental factors upon the photoperiodic response of some of the higher plants. Jour. Agr. Res. **56**: 633–677. 1938.
22. ROTH, E. R. and SLEETH, B. Butt rot in unburned sprout oak stands. U. S. Dept. Agr. Tech. Bull. 684. 1939.
23. SKOOG, F. and THIMANN, K. V. Further experiments on the inhibition of the development of lateral buds by growth hormone. Proc. Nat. Acad. Sci. **20**: 480–485. 1934.
24. SMYTH, E. M. The seasonal cycles of nitrogenous and carbohydrate materials in fruit trees. Jour. Pom. and Hort. Sci. **12**: 249–292. 1934.
25. STOECKLER, J. H. When is plantation release most effective? Jour. Forestry **45**: 265–271. 1947.
26. SWARBRICK, T. Studies in the physiology of fruit trees. I. The seasonal starch content and cambial activity in one- to five-year-old apple branches. Jour. Pom. and Hort. Sci. **6**: 137–156. 1927.
27. THIMANN, K. V. On the nature of inhibitions caused by auxin. Amer. Jour. Bot. **24**: 407–412. 1937.
28. THOMAS, W. Composition of current and previous seasons branch growth in relation to vegetative and reproductive responses in *Pyrus Malus* L. Plant Physiol. **7**: 391–445. 1932.
29. ZON, R. Management of second growth in the southern Appalachians. U. S. Forest Service Circ. 118, U. S. Dept. Agr. 1907.

THE PHYSIOLOGICAL ACTION OF SMOG ON PLANTS. I. INITIAL GROWTH AND TRANSPIRATION STUDIES

HELEN GARMAN KORITZ AND F. W. WENT

KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

Received April 21, 1952

Extensive plant damage is caused by smog (air pollutants) in the Los Angeles area. The unique symptoms, silvering and bronzing of the lower leaf surfaces and in more severe cases necrosis, have already been described by MIDDLETON *et al.* (**4**) and HAAGEN-SMIT *et al.* (**2**). The latter authors grew certain smog-sensitive plants in the Earhart Plant Research Laboratory (**6**) in smog-free air and used these indicator plants to establish the nature of the toxic substances in smog. They were able to show that the vapors of certain unsaturated hydrocarbons when combined with ozone or nitric oxide form a synthetic smog which produces plant injury identical to natural smog damage. In view of the economic importance of smog injury to plants, studies were then undertaken to determine the physiological action of synthetic smog on plants. Thus the investigations became independent of occurrence of natural smog. They include studies on the effect of environmental and certain other conditions on the growth of smog-treated plants and the influence of smog on the rate of transpiration.

Materials and methods

Visual estimation of damage proved insufficient for physiological studies, and therefore, a more quantitative method for estimation was developed. One of the symptoms of smog injury, a decrease in total growth of plants without visual damage, provided a basis for the assay, and quantitative results were obtained by measuring this hidden damage.

Uniform tomato plants (*Lycopersicon esculentum* var. San Jose Canner) were used for the assay. Seeds were planted in vermiculite approximately 3 cm. below the surface, in plastic squares measuring $10.5 \times 10.5 \times 6.5$ cm. Approximately 40 seeds were sown in each square, and the squares were placed on movable trucks in a constant temperature dark room at 26° C. From planting until harvest, the squares were watered once or twice daily with Hoagland's nutrient solution. When the seedlings broke the surface, they were moved into a greenhouse (26° C day and 20° C night) with natural light and were ultimately thinned to 10 plants per square. Two to three days prior to a test, the plants were moved to a constant temperature room with artificial light. Air in this room was filtered through a series of three carbon filters and was thus maintained completely smog-free (**2**). Temperature was kept between 20 and 22° C and the light intensity was 1000 foot-candles.

Plants 14 to 21 days old from the time of planting were used for the assay. Five, 10 or 15 squares were used for each treatment. Fumigation was carried out in a constant temperature room identical to the growing room. Synthetic smog was produced by combining ozone (0.1 p.p.m.) with vapors of 1-*n*-hexene as described by HAAGEN-SMIT *et al.* (**2**). Plants were subjected to daily fumigations and were then returned to the smog-free room. Equal numbers of untreated plants were kept in the smog-free room as controls. Unless otherwise stated, the plants were well watered just before each fumigation. As a result of preliminary tests, a one-hour fumigation performed daily for six days was chosen because it gave the greatest growth decreases. On the seventh day the plants were harvested, and the fresh and dry weights of tops and roots were recorded. In one experiment, in addition to the usual harvest data, heights and leaf areas of plants were recorded. For leaf area measurements, leaves were placed on blueprint paper between two Plexiglas plates and exposed for five minutes to light from a photoflood lamp placed about 30 cm. above the leaves. The prints were developed with cold water, dried, and the areas measured with a planimeter. Standard error of the mean was calculated for all data.

Stomatal observations were made on living plants with an Ultrapak microscope. In one case epidermal strips were also placed in vials of absolute alcohol and these strips were observed under a microscope.

Pea section tests were carried out with etiolated pea seedlings (*Pisum sativum* var. Alaska) seven days old, grown at 26° C and receiving no light except for short daily intervals of red light when the plants were watered.

Water uptake of tomato plants was measured by placing whole plants in a potometer in nutrient solution. The plants were grown as described above for assay plants; but two days before the test, the plants were removed from vermiculite, the roots washed, and the plants placed in Hoagland's nutrient solution. On the day of the test, these plants were transferred to a potometer. The stems were inserted in a rubber stopper, which had a hole approximately the size of the stem and was slit to allow placing of the stopper around the stem. Vaseline was applied in the slit and around the hole of the stopper. The roots of the plants were then immersed in the cup of the potometer which had been filled with dilute nutrient solution. During fumigation, a small telescope was used to make potometer readings through a window in the smog chamber.

Transpiration was measured by the weighing method. Individual tomato plants were grown in plastic cups in vermiculite under the conditions described above for assay plants. At the beginning of the experiment each dish was covered with four layers of wax paper fastened down by scotch tape. Holes at the base of the cups were likewise covered with scotch tape. The covers were made air tight around the plant stems by application of a mixture of paraffin wax and vaseline. Measured amounts of distilled water were added each day to bring the plants and cup back to its approximate original weight. For this a volumetric pipette was used and water was

introduced through the basal holes in the cup. Weighings were made at least four times daily on a balance modified as follows for transpiration measurements. The left pan was removed and replaced by a wire which extended through a hole in the base of the balance to an enclosed case below. A loop in the wire supported the plastic cups. Counter weights were used on the right pan.

Results

Growth studies

In order to learn more about the mode and seat of action of smog, a study was made of the effect of certain treatments on the growth response of tomato plants to smog. For these studies, unless otherwise stated, the assay method described above was used to measure the effect of the various treatments.

Initial experiments were performed on plants which had been grown according to the normal procedure already described. Results shown in table I are typical of those obtained in many such experiments. Plants

TABLE I

EFFECT OF SMOG ON THE GROWTH OF TOMATO PLANTS.
EACH FIGURE AVERAGE OF 50 PLANTS.

Treatment	Height	Leaf area	Fresh weight tops	Dry weight		
				Tops	Roots	Tops plus roots
	cm.	*cm.*^{2}	*gm.*	*gm.*	*gm.*	*gm.*
Control	14.92 ± 0.32	7.02 ± 0.12	23.02 ± 0.77	1.24 ± 0.02	0.52 ± 0.04	1.76 ± 0.05
Smog	12.54 ± 0.31	5.02 ± 0.31	16.80 ± 1.36	0.94 ± 0.07	0.42 ± 0.05	1.36 ± 0.12

fumigated daily in the mid-morning in the light displayed significant growth decreases when compared with unfumigated plants. This decrease in growth was often apparent when no other symptoms of injury, such as necrosis, silvering, or bronzing were visible and therefore can be named hidden damage. The results presented show highly significant differences between fresh weights of treated and untreated plants, but differences in dry weights which are only barely significant. In subsequent similar experiments, dry weights of tops were occasionally quite significant (table II, exps. 4 B, 5 B, and 6 A), and in other experiments were of questionable significance (table II, exps. 2 B, 3 B, and 7 A). In addition to the decrease in fresh weight, it is seen in table I that leaf areas and total heights of the plants were also reduced by smog treatment. The dry weights of roots of control and smog-treated plants consistently showed no differences.

Stomatal observations were made before, during, and after fumigation on the plants used in the above experiment. Just prior to the initial fumigation and after the plants had been subjected to several hours of light, the stomata on the upper leaf surfaces of all plants were closed; those on the lower surfaces were wide open. Stomata of plants not subjected to smog

remained the same during and for at least one hour after the period the other plants were fumigated, and each day were in approximately the same condition. Stomata of fumigated plants closed as soon as fumigation was initiated and remained closed for at least one hour after fumigation was terminated. Observations made 15 minutes after fumigation began showed all stomata of the treated plants to be closed. Before the second fumigation, the stomata of the lower surfaces of the leaves of treated plants were either closed or open only slightly. By the third day, most of the stomata on the lower surfaces of the leaves were closed before treatment. These observations were made both with the Ultrapak microscope and with epidermal strips, and further observations in similar subsequent experiments confirmed these results. Under the conditions of growth used in these studies, stomata of the upper leaf surfaces of tomatoes were always closed. In the remainder of the paper, therefore, reference to the condition of the stomata under different environments refers only to stomata of the lower leaf surfaces.

Following establishment of the above responses of tomatoes grown and treated under normal conditions, several experiments were designed to study the effect of altered environments on smog response. The first of these experiments was concerned with the effect of light, and results of these studies showed that light does affect the response of tomato plants to smog. In several experiments, each involving 100 plants per treatment, the difference in growth between plants fumigated in the dark and plants not fumigated but given the equivalent dark period was slight and insignificant. As may be seen from the data presented in table II, experiments 1, 2 A and 2 B, this difference was obtained both when the plants were fumigated immediately following the normal 12-hour dark period and when plants were fumigated following approximately one and one half hours of light. Thus in experiment 1, table II, fumigation was performed in the dark following light exposure, and differences in fresh and dry weights of control and smog plants were negligible. In experiment 2 A, table II, plants fumigated in the dark at 8 A.M. showed no decrease compared with the controls, while fumigation at 11 A.M. (table II, exp. 2 B) in the light resulted in significant decreases. In a third experiment, smog treatment was carried out at 8:30 A.M. in the light. As shown in experiments 3 A and 3 B of table II, fumigation at this hour decreased growth less than comparable fumigation at 1:30 P.M. Stomata of control plants of both groups in experiments 3 A and 3 B were closed when observed immediately after the 12-hour dark period and opened within one and one half hours after the start of the light period. On the second day of fumigation and for the remainder of the experiment, stomata of treated plants were closed.

In view of the above results, it became of interest to determine the effect of sucrose spray on the response of tomato plants to smog. An experiment was accordingly initiated in which half of the plants were sprayed daily with sucrose prior to fumigation in the light. Two sets of control plants, a sucrose-sprayed and an unsprayed series, were maintained in the smog-

TABLE II

EFFECT OF VARIOUS TREATMENTS ON THE RESPONSE OF TOMATO PLANTS TO SMOG.
EACH FIGURE IS THE AVERAGE OF 10 VALUES AND 100 PLANTS.

Experiment no.	Special treatment	Time of fumigation	Fresh weight tops		Dry weight					
					Tops		Roots		Top plus roots	
			Control	Smog	Control	Smog	Control	Smog	Control	Smog
			gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	Dark fumigation	EM*	11.84 ± 0.28	11.53 ± 0.33	0.65 ± 0.02	0.66 ± 0.02	0.36 ± 0.02	0.33 ± 0.03	1.00 ± 0.03	0.99 ± 0.04
2A	Dark fumigation	8 A.M.	42.38 ± 1.21	38.90 ± 0.96	2.37 ± 0.10	2.35 ± 0.08	0.71 ± 0.04	0.73 ± 0.03	3.08 ± 0.12	3.08 ± 0.10
2B	None	11 A.M.	42.38 ± 1.21	34.61 ± 1.40	2.37 ± 0.10	2.09 ± 0.10	0.71 ± 0.04	0.64 ± 0.04	3.08 ± 0.12	2.73 ± 0.14
3A	None	8:30 A.M.	27.80 ± 0.61	26.21 ± 0.64	1.38 ± 0.03	1.43 ± 0.04	0.49 ± 0.01	0.42 ± 0.02	1.87 ± 0.04	1.85 ± 0.05
3B	None	1:30 P.M.	27.80 ± 0.61	23.10 ± 0.86	1.38 ± 0.03	1.34 ± 0.05	0.49 ± 0.01	0.42 ± 0.02	1.87 ± 0.04	1.76 ± 0.09
4A	Sucrose spray	M**	24.58 ± 0.49	22.48 ± 0.64	1.78 ± 0.02	1.67 ± 0.01	0.55 ± 0.02	0.54 ± 0.03	2.33 ± 0.06	2.21 ± 0.06
4B	None	M**	23.41 ± 0.47	20.36 ± 0.61	1.31 ± 0.01	1.17 ± 0.01	0.44 ± 0.02	0.40 ± 0.02	1.75 ± 0.04	1.57 ± 0.04
5A	B-naphthoxy-acetic acid	M**	6.45 ± 0.20	4.14 ± 0.16	0.42 ± 0.02	0.29 ± 0.01	0.24 ± 0.02	0.15 ± 0.02	0.66 ± 0.03	0.44 ± 0.02
5B	None	M**	8.10 ± 0.17	5.31 ± 0.27	0.46 ± 0.02	0.34 ± 0.02	0.16 ± 0.02	0.12 ± 0.02	0.62 ± 0.03	0.46 ± 0.03
6A	Ample water (Group I)	M**	27.72 ± 0.71	24.53 ± 0.56	1.60 ± 0.06	1.29 ± 0.03	0.62 ± 0.03	0.50 ± 0.03	2.23 ± 0.08	1.80 ± 0.04
6B	Limited water (Group II)	M**	19.31 ± 0.47	19.35 ± 0.53	1.29 ± 0.03	1.25 ± 0.04	0.63 ± 0.03	0.55 ± 0.02	1.91 ± 0.07	1.80 ± 0.05
7A	Ample water (Group I)	1:30 P.M.	27.80 ± 0.61	23.10 ± 0.86	1.38 ± 0.03	1.34 ± 0.05	0.49 ± 0.01	0.42 ± 0.02	1.87 ± 0.04	1.76 ± 0.09
7B	Limited water (Group II)	1:30 P.M.	20.57 ± 0.30	18.42 ± 0.62	1.26 ± 0.05	1.24 ± 0.05	0.63 ± 0.03	0.56 ± 0.03	1.89 ± 0.41	1.80 ± 0.08

*EM is early morning after one to two hours of light.
**M is late morning after two to four hours of light.

free room. Solutions of 1 and 10% sucrose, each containing approximately 2 ml. per liter of Drene as a wetting agent, were used to spray plants on the first two and last four days respectively. Since no attempt was made to remove the adhering sucrose at the time of harvest, the differences between sucrose-sprayed and non-sprayed plants as seen in table II, experiments 4 A and 4 B, cannot be regarded as significant. However, the response of the sucrose-sprayed plants to smog as indicated by growth decrease was less than for plants not sprayed with sucrose. The data indicate that sucrose spray may exercise a protective action against the injurious effect of smog.

Since the effect of smog seemed to be closely related to growth, several experiments were carried out to study the effect of β-naphthoxyacetic acid (NOA) and 2,4-dichlorophenoxyacetic acid (2,4-D) on the response of plants to smog. In the first of these, tomato plants were watered with 25 ml. of a solution of NOA (50 p.p.m.) on the first two days of the six-day smog test. Treatments with NOA were discontinued after two days when the plants showed initial formative effects of the growth substance. Results of the four treatments, NOA, NOA plus smog, control, and smog, are reported in table II, experiments 5 A and 5 B. The response of the NOA-treated and untreated tomato plants to smog did not differ appreciably except in the case of the dry weights of the roots of fumigated and non-fumigated plants. A marked decrease was observed for NOA-treated fumigated plants, while in contrast there were no significant differences between the weights of the roots of fumigated and non-fumigated plants not treated with NOA.

Two further tests were carried out to determine the effect of smog on the elongation of etiolated pea stem segments treated with 2,4-D. On the seventh day after planting, half of a group of pea plants were moved to the fumigation room and were given two hours of smog treatment in the light, and half of the plants were moved at the same time into the smog-free room in the light for two hours. Immediately following fumigation, epicotyl sections were cut from uniform plants from just below the unexpanded terminal bud. Initial measurements were then made with a dissecting microscope fitted with an ocular micrometer. Ten cut sections were placed in each Petri dish containing 25 ml. of the test solution, 2,4-D (10 p.p.m.), or distilled water. Each treatment as indicated in table III was run in duplicate so that 20 sections were used for each treatment. Dishes were placed in the smog-free room for 24 hours after which the sections were again measured. As can be seen in table III, the sections treated with 2,4-D and smog were a little shorter than those treated only with 2,4-D, but the significance of the difference is questionable.

In a second pea section test, pea sections from etiolated pea plants which had not been previously fumigated or given white light were floated in open Petri dishes containing the test solutions described above. Half of these were treated with smog for two hours in the light, half were kept as controls in the smog-free room in the light. All dishes were covered after the two-hour smog period and were left for 24 hours in the smog-free room. Meas-

TABLE III

EFFECT OF SMOG ON THE RESPONSE OF ETIOLATED PEA-SECTIONS TO 2,4-D. EACH FIGURE REPRESENTS THE AVERAGE OF 10 VALUES. LENGTHS ARE IN ARBITRARY UNITS AS DETERMINED BY A NON-CALIBRATED OCULAR MICROMETER.

Treatment	Lot	Initial length		Final length	
		Control	Smog	Control	Smog
Distilled water	a	3.87 ± 0.02	3.85 ± 0.02	4.55 ± 0.04	4.56 ± 0.04
	b	3.88 ± 0.02	3.87 ± 0.02	4.61 ± 0.05	4.52 ± 0.03
2,4-D	a	3.86 ± 0.02	3.86 ± 0.02	6.31 ± 0.07	6.19 ± 0.03
	b	3.87 ± 0.02	3.86 ± 0.02	6.31 ± 0.07	6.20 ± 0.02

urements then made showed that the smog treatment did not effect the response of the sections to 2,4-D.

Observations made in the field suggested that smog damage was in some way related to the amount of water available to the plants at the time of smog attack. Damage proved to be much more severe if plants had been irrigated prior to the smog attack. These observations suggested that studies on the effect of water relations on smog damage might prove fruitful. Accordingly, tomato plants were watered once daily just before the fumigation period with measured amounts of nutrient solution. Half of the plants (group I) received an ample water supply, 100 ml. daily; half (group II) received a limited supply, 25 ml. or less daily. Plants in the latter category showed no signs of wilting for the duration of the experiment. At the termination of the experiment these plants, both control and fumigated, were smaller than the comparably treated plants of group I. In two different experiments (table II, exps. 6 A and 6 B, 7 A and 7 B), growth of fumigated plants of group II was not significantly less than control plants of the same group. However, fumigated plants of group I when compared with their controls displayed the expected decrease in growth. Stomata of control plants of group II opened each day later than those of group I and the final width attained was always less than group I. Stomata of treated plants however were uniformly closed following the first fumigation and for the duration of the experiment.

Transpiration studies

To supplement the data obtained above in studies on the effect of water relations, the effect of smog on water uptake of tomato plants was determined in two potometer experiments. Rates of water uptake of four and three tomato plants respectively were measured, and for each experiment two plants were placed in the fumigation room and one or two plants in the comparable smog-free room. After one hour, when the rates were stable, the fumigation was initiated and was continued for two hours. Readings were made for at least an hour after fumigation was discontinued. Experiment 1, using four plants, was begun at 1:30 P.M. and experiment 2, with three plants, at 11 A.M. In both experiments, marked decreases in the rate of

water uptake were observed 20 to 30 minutes after fumigation was started. In experiment 1 the rate of water uptake for treated plants dropped from original rates of 2.94 ml. per hour to 1.44 and 1.38 ml. per hour. Rates of control plants in the same interval dropped only from 4.26 and 4.20 ml. per hour to 3.60 and 3.45 ml. per hour, respectively. The results of experiment 1 are summarized graphically in figure 1 where rate of water uptake, expressed as per cent. of the original rate for each plant, is plotted against time. To obtain these values, the original stable rate of water uptake for the particular plant prior to fumigation was taken as 100, and all subsequent values were compared to this original value. Each point on the graph represents the average of three readings taken at 10-minute intervals. As can be seen in the graph, the rates of treated plants increased slightly after fumigation

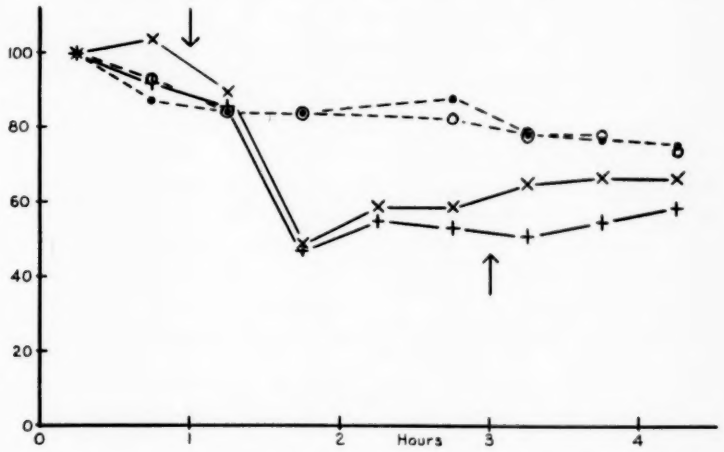

FIG. 1. Rate of water uptake (ordinate in percentage of original rate) in successive half-hour intervals. Plusses and crosses: plants fumigated for two hours with artificial smog; circles and dots: control plants not fumigated. Arrows show beginning and end of fumigation.

was discontinued but did not reach the rate for the same plant had it not been subjected to smog. Results of experiment 2 served to confirm those of experiment 1 and the same decreases in water uptake were again observed.

Subsequent measurements using the weighing method proved that the transpiration rate was also affected by smog. Transpiration rates of duplicate tomato plants given no smog, and one and two hours of smog daily for two and four days were measured. Two plants were used for each treatment except for the control in which four plants were used. A group of plants given treatments identical to those mentioned above were kept uncovered as controls for the transpiration plants in order to detect any possible differences in growth of the two groups of plants.

Following each fumigation period, a rapid drop in transpiration rate was noted. The actual values of a few typical cases are as follows: treated

plant—original rate, 2.52 ml. per hour; after first day's fumigation, 0.80 ml. per hour; after second day's fumigation, 1.09 ml. per hour. Control plant—original rate, 1.42 ml. per hour; after first day, 1.35 ml. per hour; after second day, 1.65 ml. per hour. The transpiration drop can easily be seen in figure 2 where transpiration values, expressed as per cent. of original rate of each plant are given. To obtain the values for figure 2, rates during the day were compared with the original daytime value of the particular plant (*i.e.* average rate from 9 A.M. until 1 P.M.) and rates during the night with the original nighttime value (*i.e.* average rate from 7 P.M. until 9 A.M.). Values for each control plant were also compared to the respective original values with the result that an apparent increase in transpiration rate occurred as the experiment progressed. This increase may be due to the increase in weight and surface of the plants as a result of photosynthetic activity. Fig-

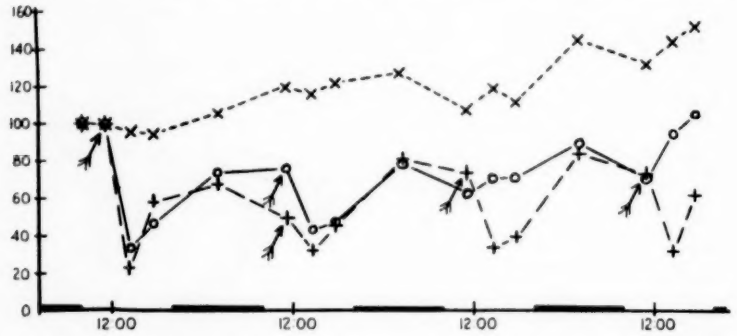

FIG. 2. Rate of transpiration (ordinate: per cent. of transpiration compared with beginning of experiment) of tomato plants kept constantly in smog-free air (crosses and broken line) or fumigated for two hours daily with artificial smog (plusses and circles). Abscissa gives time in days. Arrows indicate beginning of each fumigation. Some plants were fumigated on two successive days (circles and solid line); other plants were fumigated on four successive days (plusses and broken line).

ure 2 shows transpiration curves for a control plant and for two plants treated with two-hour daily doses of smog for two and four days. Duplicate plants showed the same trend with a marked decrease after each fumigation followed by partial recovery. Rates of plants given only two daily fumigations returned almost to their original transpiration rates by the fourth day. It is of interest that these rates for the third and fourth days parallel those of control plants but that they did not approach the values attained by the control plants. Transpiration rates of plants given only one-hour fumigations are not presented graphically but for these plants the rates likewise dropped immediately after fumigation. However the extent of the drop was slightly less than that of the two-hour fumigated plants. If one refers to figure 1, the similarity between the curve of water uptake and the first part of the curves representing transpiration in figure 2 is immediately apparent indicating that the processes measured were possibly identical for the two different methods.

Daily stomatal observations with the Ultrapak microscope were made before and after fumigations. The condition of the stomata of the covered and uncovered plants was found to be the same. Upper stomata for all plants were continually closed while the lower stomata of the control plants were wide open during the fumigation interval 1:30 to 3:30 P.M. In every case stomata of lower surfaces of smog-treated plants were closed following fumigation. Some of these reopened but on each successive day prior to fumigation more of the stomata were closed indicating permanent injury. On the fourth day most of the stomata of both one- and two-hour treated plants appeared to be completely closed. In spite of this, the typical drops in transpiration rate continued.

At the end of the experiment, all plants were harvested. Differences in dry weights and leaf area although present were not marked. Uncovered and covered plants did not differ. These results were anticipated since the number of fumigation periods was less than that required to give these differences. It was found that transpiration was affected at levels of smog lower than those necessary to give large growth differences.

Discussion

The hidden damage caused by smog is of considerable interest because it is a type of injury not known to occur in most cases of air pollution damage. In the case of SO_2 damage *e.g.* it has been clearly shown that actual damage to plants is proportional to the amount of visible leaf destruction produced by the fumigation (**5**). Invisible injury such as that caused by smog may be more widespread than is believed at present and growth of plants in many urban areas may be affected. Agents causing such damage would be insidious simply because they could cause damage not immediately apparent. In the present studies use has been made of hidden damage to measure the effect of environmental conditions and applied substances to the growth response of plants to smog. These studies were limited to the effect of synthetic smog but extension of the work to include the effect of natural smog is planned.

Fresh weights of tomato tops subjected to no other treatment than the daily smog fumigation in the light were consistently less than fresh weights of the comparable control plants even though the leaves showed no symptoms of injury. Differences in dry weights, however, varied from experiment to experiment, being one time significant and the next time not significant. These variations may possibly be reflections of slight differences in the age of the plants at the time of the initiation of the experiment. It has been observed that the extent of visible natural smog damage is dependent in part on the age of the plants, the younger plants being more sensitive. In the present studies in which plants 14 to 21 days old were used, no consistent correlation between age and hidden damage was observed, although experiments designed to test this point specifically may result in a different conclusion.

Present studies show no conclusive evidence for a positive correlation

between degree of opening of stomata and extent of damage. However it does seem that conditions favoring opening of stomata, *i.e.* light and turgidity, also are those under which plants are most susceptible to smog injury. The fact that stomata close so rapidly after first exposure to smog and remain closed often permanently would seem to indicate that stomata are not *per se* responsible for the differences in response under different treatments.

Smog injury was less severe when fumigation was carried out early in the morning either in the light or in the dark. At this time of day stomata are closed or are slightly open, photosynthesis is only commencing, and the synthetic activities related to photosynthesis have probably not yet reached their maximum rates. It is probable that plants photosynthesizing at a slow rate are less susceptible to smog damage. The results of the light experiment are similar to those obtained by KATZ and LEDINGHAM (3) and of ZIMMERMAN (9) who reported the same effects of light on sulphur dioxide injury to plants.

WENT and CARTER (8) have shown that sucrose spray can stimulate growth of tomato plants which have been placed under adverse light or temperature conditions. The sucrose may compensate for a lowered photosynthetic rate. Likewise in the present studies the sucrose spray may have compensated for a probable decrease in photosynthesis of smog-treated plants. It is also possible that the partial protective action was merely physical in that it prevented the absorption of smog.

Variations in response of oat seedlings to growth substances have been attributed by WENT (7) to the influence of air pollutants. Preliminary experiments performed in the course of the present studies failed to show a relation between smog and growth-substance activity. Soil applications of NOA solution did not alter the response of tomato plants to smog, and smog showed only a very slight and insignificant effect on the response of pea sections to 2,4-D. Since control pea sections were also unaffected, it is probable that etiolated pea plants with their small leaf surfaces absorbed quantities of smog too small to inhibit the elongation of the sections. The negative results of these experiments cannot be interpreted to mean that there is no effect of smog on growth-substance or auxin activity. The more extensive and complete experiments now in progress in this laboratory will enable a definite conclusion to be made.

The influence of water supply on injury by air pollutants is not unique to smog. A similar influence has been reported both by KATZ and LEDINGHAM (3) and ZIMMERMAN (9) who found that plants near the wilted condition were very resistant to sulphur dioxide. Plants given limited water supply are metabolizing slowly and as a result the air pollutant may be less harmful. In these studies, limiting the water also meant decreasing the amount of nutrient solution given the plant. It is unlikely that the nutrient ever became limiting, however, because of the short duration of the experiment. Since stomata closed as soon as fumigation was initiated, a drop in transpiration rate would be expected. However, even on the fourth day of

smog treatment when most stomata were closed prior to treatment, the depression in rate of transpiration was almost as great as on the first day. If smog affects transpiration simply by causing stomatal closure, it becomes necessary to account for this inconsistency. It is possible either that the stomata were very slightly open at the beginning of the fourth fumigation or that there were occasional stomata which were still open after three fumigations.

The results presented in this paper indicate that smog injury is most severe when plants are turgid, when they are in the light, and in short when conditions are optimum for photosynthesis. It is of interest that like smog, sulphur dioxide damage is also more severe when plants are in direct light, are very turgid, and when photosynthesis is at a maximum (**3, 9**). While many of the results presented here are necessarily preliminary in nature, they provide a basis from which further research on the mechanism of action of smog can proceed.

The measurement of water exchange under the influence of smog presents a curious inconsistency. Visible smog damage starts with an oily appearance of the lower leaf surface, due to leaking of cell sap from affected cells, indicating an increased permeability. The invisible injury of tomato leaves on the other hand, is associated with a decreased water uptake and transpiration, indicating decreased permeability of cell membranes. This observation would lead one to assume that the initial effect of low concentration of smog is to decrease permeability. As the smog concentration increases, this permeability effect suddenly seems to change over to an increased permeability. The smog concentration at which this change in permeability occurs must differ from plant to plant, being high for tomatoes and low for spinach and endive. When the smog concentration is raised sufficiently, tomato leaves will also show typical smog injury: browning of the lower surface and in the most severe cases yellowish-brown spots on the upper side of the leaf.

Since this article was prepared, work by BLEASDALE (**1**) has appeared in which he has shown that in Manchester, England, air pollution causes a decrease in growth rate of plants, particularly *Lolium perenne* without producing visible injury. He also shows that invisible injury was not due to sulphur dioxide. It is very likely that Bleasdale's effects were caused by the same phytotoxic agents whose affects were studied in the present investigation.

Summary

The effect of synthetic smog (1-*n*-hexene plus ozone) on growth and transpiration of tomato plants and on elongation of etiolated pea sections has been studied. Use has been made of hidden damage (growth decrease in the absence of visible injury) to measure the effect of light, sucrose spray, β-naphthoxyacetic acid, and water supply on smog injury.

Growth of tomato plants fumigated in the dark or light just following a 12-hour dark period or in the dark after one and one half hours of light was

unaffected by smog. Growth of plants fumigated in the light at mid-day or early afternoon was significantly decreased by smog treatment.

Sucrose spray partially protected tomato plants against smog damage. β-naphthoxyacetic acid did not significantly affect the response of tomato plants to smog. Smog treatment did not affect the elongation of etiolated pea sections either in water or in 2,4-D solution.

Tomato plants given limited water supply were resistant to smog damage while comparable plants receiving ample water were susceptible. Transpiration and water uptake rates of tomato plants were decreased by subjection to smog. The decrease was followed by recovery to slightly less than control rates. Subsequent fumigation caused repeated drops in transpiration rates followed by partial recovery. No direct correlation between stomatal opening and response of plants to smog was observed.

This work was supported by a grant from the Los Angeles County Air Pollution Control District. The authors wish to thank Dr. Herbert Hull for his assistance and helpful suggestions.

LITERATURE CITED

1. Bleasdale, J. K. A. Atmospheric pollution and plant growth. Nature **169**: 376. 1952.
2. Haagen-Smit, A. J., Darley, E. F., Zaitlin, M., Hull, H., and Noble, W. Investigation on injury to plants from air pollution in the Los Angeles area. Plant Physiol. **27**: 18–34. 1952.
3. Katz, M. and Ledingham, G. A. Effect of Sulfur Dioxide on Vegetation. Chapters XI, XII, XIII. Associate Committee on Trail Smelter Smoke, National Research Council of Canada. Ottawa, Canada. 1939.
4. Middleton, J. T., Kendrick, J. B., Jr., and Schwalm, H. W. Injury to herbaceous plants by smog or air pollution. Plant Dis. Reporter **34**: 245–252. 1950.
5. Thomas, M. D. and Hill, G. R. Relation of sulphur dioxide in the atmosphere to photosynthesis and respiration of alfalfa. Plant Physiol. **12**: 309–383. 1937.
6. Went, F. W. The Earhart Plant Research Laboratory. Chronica Botan. **12**: 89–108. 1950.
7. Went, F. W. The general problem of air pollution and plants. Proc. First National Air Pollution Symposium. Pp. 148–149. Stanford Research Institute, Los Angeles, California. 1950.
8. Went, F. W. and Carter, Marcella. Growth response of tomato plants to applied sucrose. Amer. Jour. Bot. **35**: 95–106. 1948.
9. Zimmerman, P. W. Impurities in the air and their influence on plant life. Proc. First National Air Pollution Symposium. Pp. 135–141. Stanford Research Institute, Los Angeles, California. 1950.

SOME RESPIRATORY CHARACTERISTICS OF THE BLUE-GREEN ALGA, ANABAENA

GEORGE C. WEBSTER [1] AND ALBERT W. FRENKEL

DEPARTMENT OF BOTANY, UNIVERSITY OF MINNESOTA, MINNEAPOLIS, MINNESOTA

Received February 27, 1952

Introduction

In recent years there has been a considerable increase in our knowledge of the respiratory metabolism in higher plants. The lower plants, however, have received little attention. This is particularly true of the blue-green algae where the amount of available information on their respiration is almost negligible. The only known investigation has been that of EMERSON (**1**) who reported that respiration in a species of Oscillatoria was inhibited about 80% by 10^{-4} M hydrogen cyanide but only 12% by a mixture of 97% carbon monoxide–3% oxygen. The purpose of this investigation has been to determine some of the over-all respiratory characteristics of a blue-green alga, and to compare certain of these characteristics with those generally found in living organisms.

Materials and methods

The alga under investigation was isolated in pure culture by R. Hecker of the University of Illinois, and identified as a species of Anabaena by Dr. E. Pringsheim of Cambridge University (**3**). The alga was grown autotrophically in an inorganic salt solution of the following composition which was modified from the Chroococcus medium of EMERSON and LEWIS (**2**): $MgSO_4 \cdot 7\,H_2O$, 0.25 gm./l.; KH_2PO_4, 0.10 gm./l.; KNO_3, 1.00 gm./l.; $Ca(NO_3)_2$, 0.025 gm./l.; NH_4Cl, 0.05 gm./l.; $FeSO_4(NH_4)_2SO_4 \cdot 6\,H_2O$, 0.004 gm./l.; Mn, 2.0 mg./l. (as $MnSO_4$); B, 1.0 mg./l. (as H_3BO_3); Zn, 0.05 mg./l. (as $ZnSO_4$); Cu, 0.01 mg./l. (as $CuSO_4$); Mo, 0.01 mg./l. (as MoO_3). Fifty-ml. aliquots of this nutrient solution were autoclaved in 250-ml. Erlenmeyer flasks, and after cooling the solution, 2.5 ml. of a Seitz filtered solution containing 1.2 gm. $NaHCO_3$ per 50 ml. were added to each flask of nutrient medium. The final pH of the medium was 9.0. The liquid cultures were inoculated from young cultures actively growing on agar slants and then grown under continuous illumination from white fluorescent lamps (at about 50 foot-candles) at a temperature of $24 \pm 1°$ C. In the course of growth the nutrient medium became more alkaline. The pH of a mature culture was about 9.5. Cultures were grown for 15 to 20 days before the cells were used for experiments. The cultures were checked periodically for contamination by bacteria and fungi by growth on glucose-peptone-agar slants.

[1] Present address: Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California.

The alga was maintained on a medium containing 2% agar in nutrient medium to which Seitz filtered $NaHCO_3$ solution was added just before the agar solidified.

Measurements of respiratory activity were carried out using the standard Warburg manometric technique. Both phosphate and carbonate buffers were used during this investigation. Dry weights of aliquots were determined for each experiment. Studies with cyanide were performed in such a way as to maintain a constant concentration of undissociated acid (**6**). Cyanide and azide were added to the test system as their sodium salts, but the concentrations of these inhibitors expressed in this paper refer to final concentrations of the non-dissociated acids, HCN and HN_3 in the Warburg vessels. All experiments with carbon monoxide were corrected for blank uptake of carbon monoxide in the presence of KOH. Appropriate controls using nitrogen-oxygen mixtures of the same percentage composition were employed during all carbon monoxide experiments. Experiments were performed at 25° C.

Fragmented cells were prepared by grinding whole cell masses at 0 to 5° C with equal quantities of 0.05 M phosphate buffer (pH 6.0) and washed Carborundum powder. The unbroken cells and the Carborundum were removed by low speed centrifugation, and the supernatant was kept at 5° C until used. Appropriate controls containing boiled fragmented cells were employed to correct for non-enzymatic oxidations.

Cytochrome c was prepared by the method of KEILIN and HARTREE (**4**).

Results

ENDOGENOUS RESPIRATION

Values for rates of oxygen consumption and for respiratory quotients are given in table I. In the absence of any added organic substrates the algal cells maintained a constant respiratory rate for several hours over a wide range of hydrogen ion concentrations and in two different buffer systems. The course of respiration was examined over an extended period of time and

TABLE I

RATES OF ENDOGENOUS RESPIRATION OF ANABAENA UNDER VARIOUS CONDITIONS OF PH AND SUSPENDING MEDIUM (DURATION OF EXPERIMENT WAS THREE HOURS).

Suspending medium	pH	Respiratory rate	Respiratory quotient
		cmm. O_2 absorbed/ mg. dry wt./h.	*cmm. CO_2 evolved/ cmm. O_2 absorbed*
Culture solution	9.3	4.5	....
0.05 M Bicarbonate-carbonate buffer (Na : K = 2 : 1)	9.1	4.6	....
0.05 M Phosphate buffer	7.9	4.7	0.90
0.05 M Phosphate buffer	7.1	4.2	0.90
0.05 M Phosphate buffer	6.0	4.6	0.90
0.05 M Phosphate buffer	5.3	4.8	0.95

showed that the initial respiratory rate continued for about four hours after the cells were placed in the dark. This was followed by a gradual decrease to a value about 20% of the original rate after 36 to 48 hours. Cells kept in the dark for three to five days became yellow but continued to respire at the low endogenous rate. They became green again after several days exposure to weak light of about 50 foot-candles. Unless otherwise indicated, results in this paper are from experiments which were performed during the first four hours after the cells had been put in the dark.

Effect of respiratory inhibitors

The heavy metal enzyme inhibitors, cyanide, azide, and *o*-phenanthroline strongly inhibited endogenous respiration (table II). It should be noted, however, that a certain portion of the respiration was not cyanide sensitive, indicating the possibility of an alternate respiratory pathway not mediated by heavy metal containing enzymes. All respiratory inhibitions, except those with *o*-phenanthroline, could be completely abolished by washing the cells several times in distilled water.

It was found that endogenous respiration was relatively insensitive to carbon monoxide until very high ratios of CO/O_2 were employed. However, the oxidation of substrate was strongly inhibited by carbon monoxide (table IV). Inhibitions were easily reversed by flushing out the manometer and vessel with air. In figure 1, the results for the inhibition of endogenous

TABLE II

INHIBITION OF OXYGEN CONSUMPTION BY INTACT CELLS OF ANABAENA BY CERTAIN RESPIRATORY INHIBITORS (CELLS IN 0.05 M PHOSPHATE BUFFER AT PH 5.5. DURATION OF EACH EXPERIMENT WAS THREE HOURS).

Inhibitor	Molar concentration	Substrate added	Range of inhibition
			%
Hydrogen cyanide*	0.00001	none	20–30
Hydrogen cyanide	0.0001	none	60–70
Hydrogen cyanide	0.001	none	70–75
Hydrogen cyanide	0.01	none	75–80
Hydrogen cyanide	0.1	none	75–80
Hydrogen cyanide	0.001	0.05 M glucose	80–85
Hydrogen cyanide	0.001	0.05 M ascorbate	85–90
Hydrogen azide	0.0001	none	0–10
Hydrogen azide	0.001	none	20–25
Hydrogen azide	0.01	none	60–70
o-Phenanthroline	0.001	none	30–40
o-Phenanthroline	0.01	none	60–65
Hydroxylamine	0.0001	none	0
Hydroxylamine	0.001	none	5–10
Hydroxylamine	0.01	none	10–20

*Inhibition of endogenous respiration by hydrogen cyanide was the same for 0.1 M to 0.00001 M HCN in carbonate-bicarbonate buffer (pH 9.1) as in phosphate buffer (pH 5.5).

respiration by carbon monoxide were plotted according to the method of LINEWEAVER and BURK (5). From this plot the distribution constant (K_D) was calculated as 29.0 (probable error $\pm$ 2.2). This value is considerably higher than the value of 2.25 obtained by WIZLER (8) for yeast respiring on glucose but is smaller than the value of 180 which can be calculated from EMERSON's data (1) for Oscillatoria. It is apparent, however, that stimulation of oxygen uptake was observed at lower CO/O_2 ratios. It remains to be determined whether this stimulation is due to oxidation of carbon monoxide or due to actual increase in the rate of oxidation of endogenous substrate. Should oxidation of carbon monoxide take place, the distribution constant calculated above would have to be corrected. For glucose, ascorbate, and hydroquinone only one CO/O_2 ratio was tested for each compound and thus

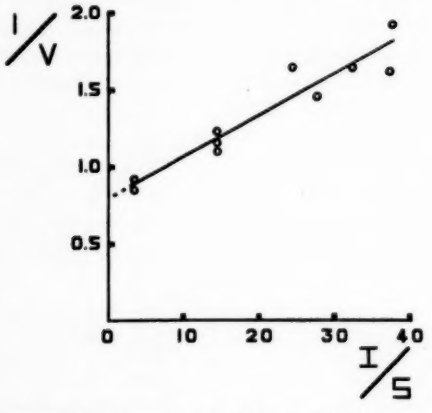

FIG. 1. The effect of different ratios of carbon monoxide to oxygen on the rate of endogenous respiration of Anabaena sp. Abscissa: Ratio of carbon monoxide to oxygen in liquid phase. Ordinate: Reciprocals of the relative rates of endogenous respiration.

only the Warburg distribution constants (7) were calculated for the observed results (table IV). The value thus obtained for glucose is also somewhat higher than those for yeast obtained by WARBURG (7) and WINZLER (8). In contrast, the oxidation of ascorbate by intact cells and of hydroquinone by fragmented cells, fortified with cytochrome c, is considerably more sensitive to carbon monoxide than the oxidation of glucose. The distribution constants in the presence of ascorbate and of hydroquinone are more comparable to those obtained by Warburg for the respiration of yeast in the presence of glucose.

An effort was made to study the effect of light on green cells whose respiration had been inhibited by carbon monoxide. The results were complicated by photosynthetic gas exchange and by an apparent inhibition of respiration in the light. The full details of these experiments will be published in a later paper. In starved cells which had lost their photosynthetic

TABLE III

OXIDATION OF ORGANIC SUBSTRATES BY INTACT AND FRAGMENTED CELLS OF ANABAENA (CELLS IN 0.05 M PHOSPHATE BUFFER AT PH 6.0. DURATION OF EACH EXPERIMENT WAS THREE HOURS. TEMPERATURE WAS 25° C).

Substrate	Oxygen uptake		Per cent. of control	
	Intact cells	Fragmented cells	Intact cells	Fragmented cells
	cmm. O_2 consumed/ mg. dry wt./hr.			
None	1.8	0.08	100	100
Glucose (0.05 M)	10.5	0.10	584	125
Galactose (0.05 M)	2.0	0.08	111	100
Mannose (0.05 M)	2.0	0.08	111	100
Succinate (0.05 M)	2.3	0.15	128	187
Acetate (0.05 M)	2.1	0.09	117	112
Pyruvate (0.05 M)	1.4	....	78	....
Catechol (0.01 M)	1.7	0.08	95	100
Ascorbate (0.05 M)	11.5	1.30	639	1625
Ascorbate + 0.0001 M cytochrome c	....	4.80	....	6000
Hydroquinone (0.01 M)	1.8	1.00	100	1450
Hydroquinone + 0.0001 M cytochrone c	....	3.60	....	4500

pigments after incubation in the dark for several days, the carbon monoxide inhibition of glucose enhanced respiration was completely reversed by light (table IV).

Oxidation of added organic substrates

Algal cells which had been kept in the dark for 24 hours to deplete photosynthetically produced substrates, were able to oxidize several organic materials (table III). In order to eliminate any influence of permeability on the oxidations, fragmented cell preparations were also used. Of interest was the finding that a polyphenol oxidase was absent from these cells since they were unable to oxidize catechol, but a cytochrome oxidase was probably present since oxidation of hydroquinone or ascorbate was enhanced by the presence of cytochrome c. The oxidation stimulated by cytochrome c was inhibited by carbon monoxide (table IV), and thus was probably mediated by a heavy metal enzyme.

Discussion

The results presented here leave little doubt that most of the respiration of Anabaena is mediated by one or more heavy metal-containing enzymes. Of the known heavy metal oxidases which have been postulated as participating in plant respiration (polyphenol oxidase, ascorbic acid oxidase, and cytochrome oxidase), polyphenol oxidase is apparently eliminated from consideration as a terminal oxidase in this organism since neither intact nor fragmented cells possessed any ability to oxidize catechol. The evidence for

TABLE IV

EFFECT OF CARBON MONOXIDE ON OXIDATIONS BY INTACT AND FRAGMENTED CELLS OF ANABAENA (CELLS IN 0.05 M PHOSPHATE BUFFER AT PH 6.0. DURATION OF EACH EXPERIMENT WAS THREE HOURS. TEMPERATURE WAS 25° C).*

Substrate	Oxygen uptake		Inhibition	Relative affinity constant
	Intact cells	Fragmented cells		
	cmm. oxygen consumed/ mg. dry wt./hr.		%	K_A
Ascorbate	11.5	....	0	....
Ascorbate + carbon monoxide ($CO/O_2 = 24.6$)*	2.5	....	78	6.9
Hydroquinone + 0.0001 M cytochrome c	....	3.60	0	....
Hydroquinone + 0.0001 M cytochrome c + carbon monoxide ($CO/O_2 = 18.2$)	....	0.90	75	6.1
Glucose (0.05 M)**	13.0	....	0	....
Glucose + carbon monoxide ($CO/O_2 = 14.4$) in the dark**	7.5	....	42	19.9
Glucose + carbon monoxide ($CO/O_2 = 14.4$) in the light**	13.2	....	0	....

*CO/O_2 in the liquid phase.
**Non-photosynthetic cells.

the occurrence of an ascorbic acid oxidase is somewhat uncertain, since the oxidation of ascorbate is stimulated by cytochrome c and would seem, therefore, to be mediated, at least in part, by a cytochrome oxidase.

On the other hand, the possibility of a cytochrome oxidase operating in respiration must be considered. The following facts point to the participation of this enzyme in the respiration of Anabaena: (a) The endogenous respiration is inhibited by cyanide, azide, and carbon monoxide; (b) The oxidation of hydroquinone and ascorbate by fragmented cells is definitely stimulated by the addition of cytochrome c and this stimulated oxidation is carbon monoxide sensitive; (c) The carbon monoxide inhibition of glucose oxidation by non-photosynthetic cells cultured in the dark is reversed by light.

Summary

The endogenous respiration of the blue-green alga, Anabaena, is independent of hydrogen ion concentration between pH 5.3 and 9.3 in phosphate and carbonate-bicarbonate buffers. When the algae are kept in the dark for two days, however, the respiratory rate declines to about 20% of the original rate.

Endogenous respiration is strongly inhibited by cyanide, azide, and *o*-phenanthroline, but is inhibited by carbon monoxide only at high ratios of CO/O_2. The calculated distribution constant is 29.

Oxidation of hydroquinone or ascorbate by fragmented cells is stimulated by cytochrome c, and the stimulated oxidation is strongly inhibited by carbon monoxide. Polyphenol oxidase is not present in Anabaena cells.

The authors wish to thank Mr. Richard Hecker for the original cultures of Anabaena and for his advice on culture methods. This study was supported by funds from the Graduate School of the University of Minnesota.

LITERATURE CITED

1. Emerson, R. Über die Wirkung von Blausaure, Schwefelwasserstoff, und Kohlenoxyd auf die Atmung verschiedener Algen. Dissertation, Friedrich-Wilhelms-Universität. Berlin. 1927.
2. Emerson, R. and Lewis, C. Photosynthetic efficiency of phycocyanin in Chroococcus and the problem of carotenoid participation in photosynthesis. Jour. Gen. Physiol. **25**: 579–595. 1942.
3. Hecker, R. C. Personal communication.
4. Keilin, D. and Hartree, E. F. Purification and properties of cytochrome c. Biochem. Jour. **39**: 289–292. 1945.
5. Lineweaver, H. and Burk, D. The determination of enzyme dissociation constants. Jour. Amer. Chem. Soc. **56**: 658–666. 1934.
6. Robbie, W. A. The quantitative control of cyanide in manometric experimentation. Jour. Cell. and Comp. Physiol. **27**: 181–210. 1946.
7. Warburg, O. Über die Wirkung von Kohlenoxyd und Stickoxyd auf Atmung und Gärung. Biochem. Zeit. **189**: 354–380. 1927.
8. Winzler, R. A comparative study of the effects of cyanide, azide, and carbon monoxide on the respiration of bakers' yeast. Jour. Cell. and Comp. Physiol. **21**: 229–252. 1943.

THE METABOLISM OF FOLIAR-APPLIED UREA. I. RELATIVE RATES OF $C^{14}O_2$ PRODUCTION BY CERTAIN VEGETABLE PLANTS TREATED WITH LABELED UREA[1]

O. N. HINSVARK, S. H. WITTWER AND H. B. TUKEY

DEPARTMENT OF HORTICULTURE, MICHIGAN STATE COLLEGE,
EAST LANSING, MICHIGAN

Received June 20, 1952

Introduction

A number of horticultural crops respond favorably to foliar applications of urea. Considerable variability exists among crops, however, in the tolerances of the foliage to spray concentrations of urea as well as in the apparent rate of utilization, and in the growth and fruiting responses obtained (**2, 3, 4**). Plants showing the least tolerance to urea, in general, respond most favorably to physiologically tolerable amounts of it. The greater effectiveness of the nitrogen in urea when foliar-applied, as compared with the usual inorganic ionic forms, is generally believed to reside in its non-polar organic properties. The first step in the utilization of the nitrogen in urea by the leaves of plants presumably is hydrolysis by the enzyme urease giving ammonia as one of the products.

$$\begin{matrix} NH_2 \\ \diagup \\ C = O \\ \diagdown \\ NH_2 \end{matrix} + H_2O \xrightarrow{\text{urease}} 2\ NH_3 + CO_2$$

In view of the almost immediate responses obtained with urea sprays on a number of crops, this reaction must proceed rapidly, and one of the factors limiting the reaction rate may be the urease activity in the leaves. Furthermore, rapid hydrolysis, possibly induced by high urease activity, may cause the accumulation of hydrolytic products conducive to the foliage injury easily achieved on most crops but which varies among species with regard to the highest concentration which can be applied without visual burning of the foliage.

As a measurement of the urease activity, and, similarly the rate of hydrolysis and possible utilization of urea applied to the leaves of several vegetable plants, carbon-14-labeled urea was employed, and the relative rates of the evolution of radioactive carbon dioxide were determined.

Materials and methods

Cucumber, bean, tomato, sweet corn, celery, and potato plants were grown to comparable sizes in sand in four-inch clay pots with complete

[1] Journal article no. 1380 of the Michigan Agricultural Experiment Station.

nutrients in the greenhouse. Normal plants four to six inches in height and growing vigorously were selected for treatment.

A weighed quantity of C^{14} urea was dissolved in 0.25 to 0.5 milliliters of a 0.3% Dreft solution. The amount of solvent used was dependent upon the ability of the leaf surface to retain the solution. The urea concentrations varied from 0.1 to 0.5%, but were always held below the predetermined concentration considered toxic when applied as a spray to the leaves of a particular plant. The C^{14} urea was purchased from the Los Alamos Scientific Laboratory and had a specific activity of 1 mc. per 18 milligrams. Measured quantities of the solutions were applied immediately after preparation to a given area on the upper surface of the leaf or leaves of individual plants. An attempt was made to have the total area of application as well as the physiological age of the leaves of the various species as constant as

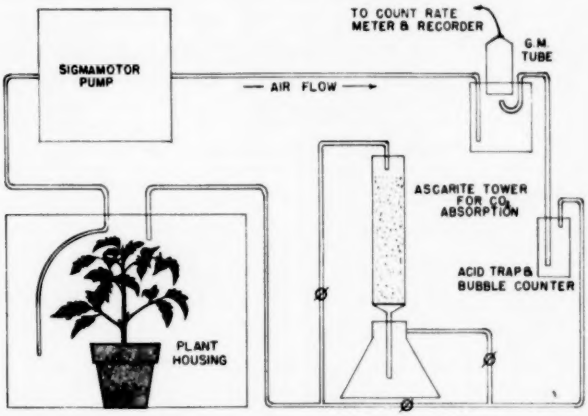

FIG. 1. Schematic drawing of closed system used to measure accumulation of $C^{14}O_2$ produced by hydrolysis of C^{14} urea.

possible. Young leaves, not fully expanded, were always selected for treatment. Before treating, all plants were held in the dark for at least one hour. Initial tests suggested that such a preconditioning greatly minimized the possible dark fixation of CO_2.

Following treatment and during the measurement of hydrolysis of urea the plants were held in the dark in order that the $C^{14}O_2$ evolved would not be re-utilized in photosynthesis. Several (never less than three) replicate runs were made on a single species to determine the pattern of hydrolysis including the approximate rate and kinetic order of the reaction.

The comparative rates of accumulation of $C^{14}O_2$ as a result of urea hydrolysis by the leaves of the various plants were measured by the apparatus shown in figure 1. A Nuclear Instrument and Chemical Corporation count rate meter (model 1615A) with an attached Esterline-Angus A. W. recorder was used in the design. For uniform voltage output for the count

rate meter, a Solavolt constant voltage transformer was connected in the circuit. To provide a slow circulation of the atmosphere, which was continuously monitored in the closed system, a Sigmamotor pump controlled by a Boston Gear Works reductor (model LW13) was found satisfactory. This combination circulated the enclosed volume at a slow rate so that a representative sample was measured by the count rate meter and recorded by the attached recording instrument at all times. A typical activity curve thus obtained showing the accumulation of $C^{14}O_2$ in the system subsequent to the application of 10 μc. of C^{14} urea to the leaf of a tomato plant is illustrated in figure 2A. A Nuclear Instrument and Chemical Corporation radiation counter with a D-34 mica window having a thickness of 1.4 mg./cm.^{2} was used. Ten microcuries of $C^{14}O_2$ urea when applied to a leaf in the typical manner gave from 200 to 500 counts per minute with this apparatus. Neoprene tubing was employed throughout the system since it was found superior to natural rubber in withstanding the constant flexing exerted by the pump and because it has a relative impermeability to diffusion of CO_2. The acid trap provided a means of collecting ammonia evolved into the system

TABLE I

PLANT TOLERANCES TO CONCENTRATIONS OF UREA.

Plant	Pounds of urea per 100 gallons of water
Cucumber	3–4
Bean	4–5
Tomato	4–6
Corn	4–6
Celery	8–14
Potato	8–14

during the course of urea hydrolysis, and following each experimental run this solution was always checked by Nessler's reagent for the presence of ammonia. At the conclusion of each experiment the entire enclosed volume was shunted through the ascarite tower for CO_2 absorption.

Urea toxicity tests were conducted under greenhouse conditions on 10 plants of each of the six species. Plants of the same age and size and which had been grown under similar conditions of temperature, sunlight, mineral nutrition, and moisture to those used in the C^{14} urea studies were selected for treatment. Concentrations of urea ranging from 2 to 20 pounds per 100 gallons of water with Dreft added as a wetting agent were applied by momentarily dipping the tops of the plants into the various solutions. Treatments were applied three times at weekly intervals. In table I the lowest concentrations causing some marginal leaf burning are listed for each crop.

Experimental results and discussion

The comparative rates of hydrolysis of C^{14} urea by the leaves of bean, corn, tomato, and cucumber plants as determined by the accumulation of

$C^{14}O_2$ in the closed system were recorded. In figure 2 A is presented a typical activity-time curve for the hydrolysis of urea applied to the leaves of a tomato plant and the portion of the curve used for determining its slope and rate constant. The slopes of similar curves are portrayed, and rate con-

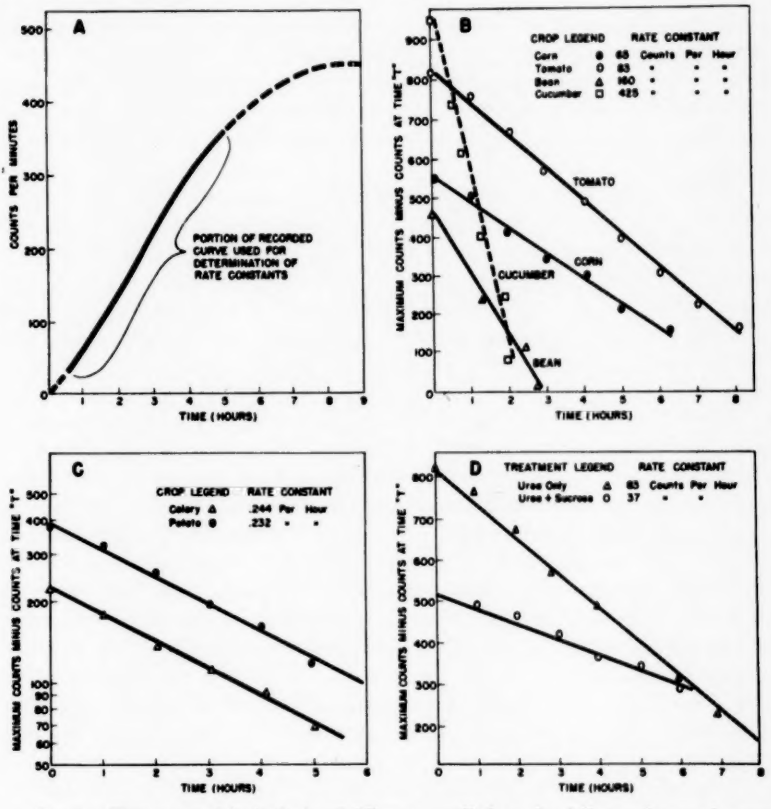

FIG. 2. The rates of hydrolysis of C^{14} urea applied to the foliage of several vegetable plants as illustrated by the initial slopes of the activity-time curves, showing the accumulation of $C^{14}O_2$ in a closed system. A. A zero order activity-time curve showing the approximate portion of the recorded curve used for determination of rate constant. B. Typical zero order rate constants and curve slopes for corn, tomato, bean, and cucumber. C. First order rate constants and slopes for celery and potato. D. Comparative rate constants and curve slopes for the tomato with urea only and with urea and sucrose.

stants are listed in figure 2 B for cucumber, bean, tomato, and corn plants. The reaction rates for these four plants were typically of zero order. The most rapid rate of hydrolysis occurred when urea was applied to the cucumber leaf, followed by the bean, tomato, and sweet corn. Within the range of concentrations used in these tests, the calculated rate constants were found

to be relatively independent of the amount of urea originally applied to the leaves, and definite slopes of the curves were found to be characteristic for a given species and easily reproducible.

It is of interest to note that within two to three hours subsequent to treatment, foliar-applied urea apparently is completely hydrolyzed by cucumber leaves, and of all plants tested the leaves of the cucumber exhibit the greatest urease activity. This is of further significance since observations of tolerance to spray concentrations reveal (table I) that cucumber leaves are the most sensitive of the six plants tested; they are injured by concentrations greater than three to four pounds of urea to 100 gallons of water.

The rapid hydrolysis of urea by bean leaves (variety, Black Valentine) and their sensitivity to urea (table I) is not surprising in that urease was first crystallized from Jack bean meal (**5**). The tomato and sweet corn plants show considerably lower rate constants, but it is significant, even with these plants, that the hydrolysis of foliar-applied urea proceeds rapidly, that within a few hours following application the reaction is complete, and that tomato and sweet corn plants are relatively sensitive to low concentrations of urea.

The leaves of celery and potato were found to be much more tolerant to successive sprays of urea (table I). The hydrolyses of urea as measured by the evolution of $C^{14}O_2$ from the C^{14} urea applied to these leaves were found to have different reaction rates and gave straight lines when the initial log counts were plotted against time (fig. 2C). The kinetics of such reactions are of first order, and the rate constants suggest a remarkable similarity in the urease activities of the foliage of celery and potato. The low rate constants for urease activity may indicate a basis for the high tolerance of the foliage of these crops to urea sprays. At low concentrations of urea (0.02%) applied to the leaves of celery and potato, the kinetics appeared to be of zero order with a rate constant of approximately 20. It is possible with these plants that the amount of the enzyme rapidly becomes a limiting factor as the reaction proceeds.

Tolerances of the leaves of plants to urea as related to the time-activity curves, which are in turn determined by the hydrolysis rate constants, are of special interest in view of the report that sucrose applied in equal molar quantities to urea protects the foliage of the tomato plant against possible injury by urea sprays (**1**). A solution containing 0.1 molar sucrose and C^{14} urea was prepared, applied in the usual manner, and the rate constant was derived from the initial slope of the time-activity curve and compared with that characteristic of the usual application of C^{14} urea on the leaves of the tomato plant. These data are presented in figure 2D. As was characteristic of the tomato, both reaction rates were typically zero order, but urea hydrolysis was significantly reduced by the addition of sucrose in equal molar quantities. Possibly sucrose exerts its effect in preventing foliage burning of the tomato by partially inhibiting urease activity, which may in

turn prevent the rapid accumulation of toxic hydrolytic products of the reaction. In practice this effect of sucrose may be advantageous in that higher concentrations of urea may be applied and urea utilization extended for a longer period of time. Similarly, the immediate and characteristic responses from nitrogen applications to leaves may not be so readily apparent with urea if sucrose is added.

Of special significance in these studies appears to be the relationship apparently established between the reaction rates characteristic of urea hydrolysis by the leaves of various plants, the growth responses of these plants to urea and the concentrations of urea which can be applied without burning injury to the foliage. The data reported would suggest that plants most easily injured by urea sprays are apparently those which have the highest urease activity in the leaves. Conduciveness toward injury is indicative of rapid hydrolysis denoting rapid utilization and distinguishes the plants most readily benefited if below-toxic concentrations are employed. With the plants studied, urea hydrolysis as it occurs in the leaves of plants subsequent to foliar applications, appears to be the equivalent of utilization. In no instance was any ammonia collected in the acid trap (fig. 1) during the course of urea hydrolysis in the atmosphere of the closed system employed in these tests.

Summary

The first step in the utilization of the nitrogen in urea applied to the leaves of plants presumably is hydrolysis by the enzyme urease giving NH_3 and CO_2. The comparative rate constants of the activity-time curves of the enzymatic hydrolysis of carbon-14-labeled urea applied to the leaves of cucumber, bean, tomato, corn, celery, and potato were determined by continuously monitoring the air and recording the accumulation of $C^{14}O_2$ in a closed system.

The initial reaction rates for cucumber, bean, tomato, and corn, the leaves of which were found relatively intolerant to urea, were typically of zero order. With celery and potato plants having considerably more tolerance to foliar-applied urea, the kinetics of the enzymatic hydrolysis of urea by the leaves were of first order. Equal molar concentrations of sucrose used in solution with urea and applied to the foliage of tomato plants significantly reduced the rate of urea hydrolysis.

It is suggested that plants most easily injured by urea sprays are apparently those which have the highest urease activity, and the initial kinetics of the reaction are of zero order. Conduciveness toward urea injury is indicative of rapid hydrolysis and may denote rapid utilization. Hydrolysis as it occurs in the leaves of plants subsequent to foliar applications of urea appears to be equivalent to utilization.

This work was supported by a grant from the Atomic Energy Commission, Biological and Medical Division, Contract At(11-1)-159. Suggestions of F. E. Kelsey of the Nuclear Instrument and Chemical Corporation, Chi-

cago, were utilized in designing the apparatus (fig. 1) for continuously monitoring the accumulation of $C^{14}O_2$ in a closed system.

LITERATURE CITED

1. Emmert, E. M. and Klinker, J. E. Spraying tomato foliage with sucrose to increase carbohydrates and protect against injury by urea sprays. Kentucky Agr. Exp. Sta. Bull. 550. 1950.
2. Fisher, E. G. and Cook, J. E. Nitrogen fertilization of the McIntosh apple with leaf sprays of urea II. Proc. Amer. Soc. Hort. Sci. **55**: 35–40. 1950.
3. Mayberry, B. D. and Wittwer, S. H. Some results with urea-nitrogen applied to the leaves of certain vegetable crops. Michigan Quart. Bull. 1952.
4. National Joint Committee on Fertilizer Application. Proc. 27th Annual Meeting. Pp. 37–51. 1951.
5. Sumner, J. B. and Somers, G. F. Chemistry and Methods of Enzymes, Second Edition. Academic Press, New York. 1947.

THE RELATION OF THE AMINO ACID COMPOSITION TO THE DEVELOPMENT OF OATS

THOMAS A. McCOY, THOMAS H. SUBLETT AND VERGIL W. DOBBS
THE SAMUEL ROBERTS NOBLE FOUNDATION, ARDMORE, OKLAHOMA

Received April 4, 1952

Introduction

The phenomenon of photoperiodism has held the interest of investigators for a number of years. The fact that one environmental factor can exert such influence on the sexual development of some plants has been responsible for many investigations. The results of most of this work have been excellently reviewed elsewhere (**11, 16**) and will not be discussed here. However, the authors were unable to find the results of any extensive investigation of plant development when the chronological age of the plant was held constant. It seemed that the composition of plants having the same chronological age but different physiological ages should shed some light on this phenomenon. This paper is a record of work that was designed to investigate the relation between the amino acid content and the development of the oat plant (a long-day plant).

Experimental procedure

The test plant used was Wintok Oats CI-3424, furnished by the Oklahoma Agricultural Experiment Station, Stillwater, Oklahoma. The grains, which had been treated with Ceresan, were germinated on wet paper towels and transplanted to four-inch glazed soil jars when the plumules had emerged from the hull. The jars were filled with a mixture of clay loam soil, sand, barnyard manure, and some commercial fertilizer. This mixture was prepared in one large vat and thoroughly mixed before being placed in the pots. Soil was used as a medium for growing the plants, since it is generally accepted that variations of light affect the organic composition of plants much more than variations in inorganic nutrients available to the plants (**7, 12, 13, 15**).

When the seedlings were one week old, they were thinned to three plants per pot. There were five pots per treatment and a total of one hundred pots were started for the experiment. The pots were kept in darkness until the seedlings emerged from the soil. At that time all of the pots were placed in an 8-hour day. Each week five pots were transferred from the 8-hour photoperiod to a 16-hour photoperiod in order to induce development. This procedure was used so that at the end of the experiment all of the plants would have the same chronological age but would be in varying stages of physiological development from the purely vegetative stage up to and including complete exsertion and anthesis. For the purposes of this paper, the

different treatments will be referred to in the following manner. Group 0 contained the plants which remained in the 8-hour photoperiod for the duration of the experiment. Group 1 consisted of the plants which had been exposed to two 16-hour photoperiods just previous to the termination of the experiment. Groups 2 through 10 were exposed to a 16-hour photoperiod for successively longer periods in increments of one week. Thus, group 2 was exposed to 61 8-hour days followed by nine 16-hour days, and group 10 was exposed to seven 8-hour days followed by 65 16-hour days.

The seeds were germinated on October 27, 1950, and transplanted to the soil jars four days later. On November 1, 1950, the seedlings had emerged from the soil. On November 12, the plants started tillering, but no significant differences in the time or amount of tillering were noticed between

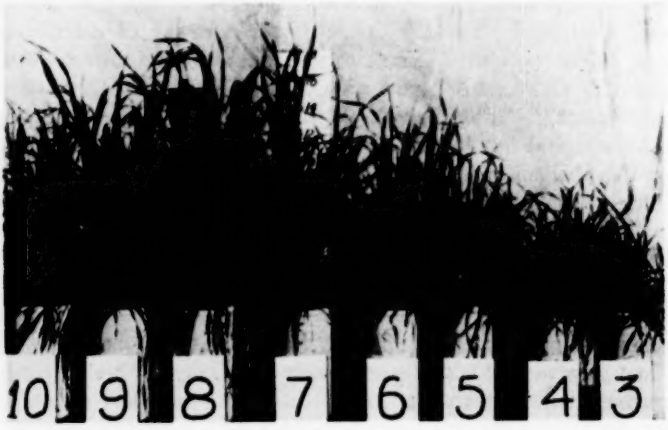

FIG. 1. Oats, two months old, in various stages of development. Groups 8 and 9 were further developed than group 10 which was exposed to a shorter preliminary period of eight-hour lengths. Numbers refer to the physiological age of the plant (see text).

treatments. When the plants were 26 days old, the oats in group 10 began to shoot and four days later the plants in group 9 started shooting. Plants of group 8 did not begin to shoot until eight days later. When the plants were two months old, it was noted that those in the eighth and ninth treatments were definitely taller than the plants in group 10. These findings were similar to those of PURVIS and GREGORY (18) who demonstrated in winter rye (a long-day plant) that flowering was accelerated when the unvernalized plants were exposed to a preliminary period of short-day lengths. Figure 1 shows the plants at this stage.

On January 2, 1951, three plants in group 9 had flowered, and the following day five plants in group 8 had flowered. By January 5, all of the plants in group 9, nine plants in the eighth group, and two in the tenth group had flowered. By January 6, the remaining plants in group 8 had flowered. On January 7, all of the plants in groups 8, 9, and 10 had flowered, and plants

in four pots of the seventh treatment were in flower. By January 10, all the plants in groups 6 through 10 had flowered (fig. 2).

On January 12, exsertion was complete in the eighth and ninth groups. Before all the oat panicles appeared, anthesis had occurred in the terminal panicles. This development was characteristic throughout all the treatments which induced flowering. On January 13, the experiment was terminated. Harvesting consisted of separating the panicles, stems, leaves (including sheaths), and the roots into four separate groups. The roots were washed with saturated saline solution to remove the adhering soil particles. The samples were dried in an oven for eight hours at 70° C. Afterwards the temperature was increased to 100° C for 16 hours. The plant material was allowed to equilibrate with the moisture in the air, and air-dried weights were taken. The samples were ground in a small Wiley mill using a 60-mesh

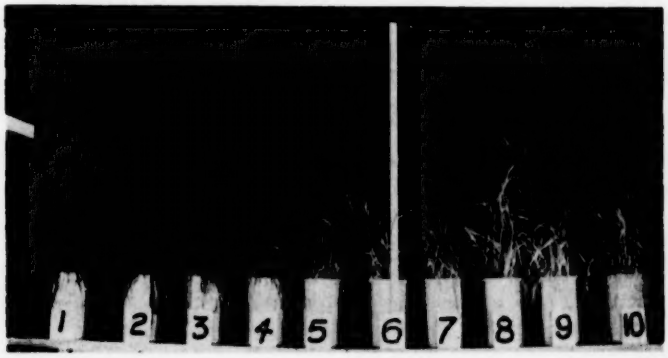

FIG. 2. Oats at the termination of the experiment. All plants were the same chronological age but varied in physiological age from the vegetative stage up to complete exsertion (groups 8 and 9). Numbers refer to the physiological age of the plant (see text).

sieve. Because of the difficulty of removing all the roots from the soil, no dry weights were made on these structures. The ground tissue was composited, and samples were weighed as air-dried material.

At the time of harvest, several plants in groups 0, 1, and 2 were dissected to determine the stage of the growing point. The plants which remained in the eight-hour day during the complete duration of the experiment had apparently not developed further than an early transition stage. Comparisons of the growing points were made with the photographs of BONNETT (3). The oats remaining in the eight-hour photoperiod were in a vegetative stage or a very early transition stage. The tip of the main axis had elongated in some cases but the branch initials (first order) were not apparent.

The group 1 plants had been exposed to two 16-hour days, and it was interesting to note that all of these plants examined corresponded to BONNETT'S plates C and D which represented the first stage of panicle formation

and the formation of branches of the second order. The plants which were in a favorable photoperiod for 10 days were developed much further.

Methods of analysis

For the analysis of tryptophan, the air-day plant material was hydrolyzed with 5 *N* NaOH. This procedure consisted of autoclaving the samples at 15 pounds of pressure for five hours. The hydrolyzates for the remaining amino acids were prepared by hydrolyzing the plant material with 6 *N* HCl for 18 hours.

Glutamic acid, leucine, phenylalanine, valine, and tryptophan were determined with *Lactobacillus arabinosus* 17-5; arginine and threonine by *Streptococcus faecalis* R; aspartic acid, histidine, tyrosine, isoleucine, and proline with *Leuconostoc mesenteroides* P-60. All of these methods were described by Henderson and Snell (**8**). Lysine and serine were determined by the microbiological procedure of Cardinall and Hedrick (**4**). Glycine was assayed by the method of McCoy and Wender (**14**). Cystine and methionine were determined by the method of Sauberlich and Bauman (**19**).

Results and discussion

To avoid confusion the amino acids are considered in five principal groups: the basic group which includes arginine, histidine, and lysine; the acidic group which includes glutamic acid and aspartic acid; the aromatic and heterocyclic group which includes phenylalanine, proline, tyrosine, and tryptophan; the aliphatic group which includes glycine, serine, threonine, valine, leucine, and isoleucine; and the sulphur-containing amino acids, cystine and methionine. The amounts of the amino acids will be referred to in micromoles of the L-isomer.

Concentration of the amino acids

The tabular data on the concentration of amino acids on a dried weight basis were omitted from the text for the sake of brevity, but this information can be derived from the dried weight data in table I and tables III, IV, V, and VI, if desired. The exception of this rule was the root data, since the total weights of the roots were not determined.

Some general trends were noted with respect to amino acid concentration. On the basis of micromoles per gram dry weight, the concentration of

TABLE I

DRIED WEIGHTS OF OAT PLANTS AS GRAMS OF AIR-DRIED MATERIAL.

Plant parts	Group										
	0	1	2	3	4	5	6	7	8	9	10
Leaves	4.70	6.14	6.93	7.41	7.48	8.82	13.17	19.12	14.46	13.62	13.45
Stems					3.45	5.43	7.73	8.25	9.68	9.82	9.58
Fruits							.98	3.95	4.76	3.90	4.24

most of the amino acids decreased in the leaves until the time just previous to flowering. After the stages reached in groups 5 and 6, there was an increase in concentration of most of these compounds. The concentration of tryptophan remained exceptionally constant in the leaves during the complete developmental cycle. It should be mentioned that there was an extremely low concentration of tryptophan in group 0 as compared to the rest of the plants.

The concentration of most of the amino acids in the roots increased from group 0 to group 6 (see table II). This increase was followed by a decrease in concentration. Apparently the roots were building up a reserve of amino acids in the earlier stages of development, and when the flowers appeared, these compounds were rapidly translocated into the tops. Glutamic acid

TABLE II

CONCENTRATION OF AMINO ACIDS IN OAT ROOTS IN MICROMOLES PER GRAM DRY WEIGHT.

Amino acids	Group										
	0	1	2	3	4	5	6	7	8	9	10
L-Arginine	27	29	29	37	36	41	39	48	36	39	36
L-Histidine	5.5	6.0	5.7	6.0	5.5	7.2	6.7	8.4	5.7	5.7	7.2
L-Lysine	20	21	20	25	23	30	26	41	22	26	20
L-Glutamic acid	41	38	35	39	35	47	51	41	34	46	40
L-Aspartic acid	27	26	23	20	23	27	32	35	29	31	29
L-Phenylalanine	24	26	25	26	26	30	33	36	26	32	30
L-Tyrosine	8.3	6.1	6.1	7.7	8.3	9.1	11.0	9.9	8.3	10.5	8.3
L-Proline	20	20	17	17	17	24	28	28	19	17	18
L-Tryptophan	6.9	8.8	10.8	8.8	10.8	8.8	5.9	13.7	4.4	8.8	10.3
Glycine	39	41	41	44	41	48	55	48	27	43	40
L-Serine	9	12	20	22	19	23	26	19	20	25	27
L-Threonine	21	23	23	26	26	27	34	26	23	32	32
L-Valine	24	27	30	30	30	36	36	38	23	35	29
L-Leucine	31	21	31	31	31	26	28	30	21	28	28
L-Isoleucine	27	25	23	26	26	27	30	32	25	29	27
L-Cystine	5.4	5.8	8.3	5.0	6.7	6.7	7.1	5.4	5.8	4.6	8.3
L-Methionine	4.0	4.3	3.4	5.0	5.4	7.0	7.4	7.0	5.4	7.4	7.9

and aspartic acid presented a similar picture except in the initial stages of development (groups 0 to 4) when there was a decrease in concentration of these two compounds in the roots. Since there was an increase in the total amount of glutamic acid and aspartic acid in the leaves at this time, it appeared that the acidic group was translocated rapidly from the roots to the leaves in this stage of development.

During the rapid elongation of the stems (groups 4 to 6), most of the amino acids exhibited a decrease in concentration in the stems (probably a dilution factor). In plants of groups 7 to 10 there was an increase in concentration. Generally speaking, there was an increase in the concentration of amino acids in the fruits as they matured. The concentration of the aromatic and heterocyclic compounds and the aliphatic group (except isoleucine) increased. The concentration of the sulphur-containing amino acids

TABLE III

TOTAL AMOUNT OF AMINO ACIDS IN OAT LEAVES IN MICROMOLES PER GRAM DRY WEIGHT.

Amino acids	Group										
	0	1	2	3	4	5	6	7	8	9	10
L-Arginine	290	300	330	320	200	310	550	730	670	670	550
L-Histidine	130	150	130	130	90	110	310	270	260	250	190
L-Lysine	500	600	600	600	550	450	750	1300	1000	950	450
L-Glutamic acid	880	1100	1160	1410	1120	840	590	1720	1450	1360	1350
L-Aspartic acid	470	580	590	590	520	490	650	1000	810	760	750
L-Phenylanaline	660	800	750	650	650	650	1300	1900	1300	1250	1000
L-Tyrosine	110	150	120	130	180	200	250	340	320	270	270
L-Proline	310	390	360	370	590	690	760	750	350	330	280
L-Tryptophan	10	35	30	35	35	40	60	95	65	45	65
Glycine	800	900	900	900	900	950	1400	2100	1700	1600	1700
L-Serine	560	540	490	480	450	440	530	840	620	590	480
L-Threonine	390	520	530	590	410	490	710	1200	950	860	710
L-Valine	520	470	690	650	620	570	820	1500	1100	1100	970
L-Leucine	420	500	510	470	470	510	910	1100	1000	980	920
L-Isoleucine	440	490	510	520	410	500	790	1100	870	820	650
L-Cystine	45	60	70	70	75	95	200	210	120	130	110
L-Methionine	15	30	10	15	35	55	170	140	110	150	110

and arginine remained constant as the fruits matured while isoleucine decreased in the fruits from groups 7 through 9.

TOTAL AMOUNT OF AMINO ACIDS

The total amount of amino acids in the leaves, stems, fruits, and plant tops are listed in tables III, IV, V, and VI. Most of the amino acids remained fairly constant in the plant top during the early stages of develop-

TABLE IV

TOTAL AMOUNT OF AMINO ACIDS IN OAT STEMS IN MICROMOLES

Amino acids	Group						
	4	5	6	7	8	9	10
L-Arginine	80	80	120	180	170	190	150
L-Histidine	40	50	50	60	70	90	100
L-Lysine	150	200	150	250	250	300	200
L-Glutamic acid	760	780	820	910	1360	1770	1150
L-Aspartic acid	310			550	680	820	630
L-Phenylalanine	150	200	200	300	300	350	250
L-Tyrosine	40	50	60	60	70	50	60
L-Proline	160	290	270	310	280	290	280
L-Tryptophan	10	10	15	25	30	25	25
Glycine	200	300	250	400	350	350	400
L-Serine	120	140	240	240	310	360	310
L-Threonine	150	230	160	210	290	340	330
L-Valine	210	280	210	350	380	420	350
L-Leucine	170	200	180	250	250	330	270
L-Isoleucine	90	180	240	310	270	330	200
L-Cystine	25	40	75	65	75	75	70
L-Methionine	10	20	25	30	25	25	35

TABLE V

TOTAL AMOUNT OF AMINO ACIDS IN OAT FRUITS IN MICROMOLES.

Amino acids	Group				
	6	7	8	9	10
L-Arginine	40	160	220	180	180
L-Histidine	10	50	60	70	60
L-Lysine	50	250	250	300	250
L-Glutamic acid	110	470	520	470	470
L-Aspartic acid	60	270	230	280	290
L-Phenylalanine	50	300	300	350	300
L-Tyrosine	20	40	60	90	80
L-Proline	50	190	270	300	310
L-Tryptophan	5	10	15	15	10
Glycine	100	350	450	450	350
L-Serine	40	170	260	170	170
L-Threonine	50	200	260	250	240
L-Valine	60	270	320	300	290
L-Leucine	50	230	290	240	240
L-Isoleucine	50	200	210	170	170
L-Cystine	15	55	55	60	40
L-Methionine	5	30	35	30	35

ment (groups 0 to 5). After the rapid stem elongation, these amino acids exhibited a definite increase in the plant top. Lysine showed the largest increase of the basic group increasing from around 700 micromoles in groups 4 and 5 up to 1500 micromoles in groups 8 and 9.

There was a definite increase in the tryptophan content between groups 0 and 1 which is shown in figure 3. Since there was a decided difference between the growing points of the plants in group 0, which were in a very

TABLE VI

TOTAL AMOUNT OF AMINO ACIDS IN OAT TOPS IN MICROMOLES

Amino acids	Group										
	0	1	2	3	4	5	6	7	8	9	10
L-Arginine	290	300	330	320	280	390	710	1070	1060	1040	880
L-Histidine	130	150	130	130	170	190	370	380	390	410	350
L-Lysine	500	600	600	600	700	650	950	1800	1500	1550	900
L-Glutamic acid	880	1100	1160	1410	1880	1620	1520	3100	3330	3600	2970
L-Aspartic acid	470	580	590	590	830	490	710	1820	1720	1860	1670
L-Phenylalanine	660	800	750	650	800	850	1550	2500	1900	1950	1550
L-Tyrosine	110	150	120	130	220	250	330	440	450	410	410
L-Proline	310	390	360	370	750	980	1080	1250	900	920	870
L-Tryptophan	10	35	30	35	45	50	80	130	110	85	100
Glycine	800	900	900	900	1100	1250	1750	2850	2500	2400	2450
L-Serine	560	540	490	480	570	580	810	1250	1190	1120	960
L-Threonine	390	520	530	590	560	720	920	1610	1500	1450	1280
L-Valine	520	470	690	650	830	850	1090	2120	1800	1820	1610
L-Leucine	420	500	510	470	640	710	1140	1575	1540	1550	1430
L-Isoleucine	440	490	510	520	500	680	1080	1610	1350	1320	1020
L-Cystine	45	60	70	70	100	135	290	330	250	265	220
L-Methionine	15	30	10	15	45	75	200	200	170	205	180

early transition stage, and the growing points of the plants in group 1, which exhibited the branch initials (second order) it appeared that tryptophan plays an important role at the time of initiation of the flower primordia. It is well known that indoleacetic acid and other growth substances can suppress flowering in many plants (**1, 6, 10, 17**). BONNER and THURLOW (**2**) demonstrated that certain auxin antagonists can initiate flower-like buds in Xanthium when the plants are growing in a 16-hour day. The conversion of tryptophan into indoleacetic acid has been reported to occur in etiolated pea seedlings by GALSTON (**5**). This conversion involved the decarboxylase and transaminase systems to produce indolepyruvic acid or tryptamine from tryptophan, and either of these two compounds were converted to indoleacetaldehyde. The subsequent oxidation of indoleacetaldehyde would yield indoleacetic acid. The present experimental evidence supports these findings in that the oat plants in group 1 contained much larger amounts of

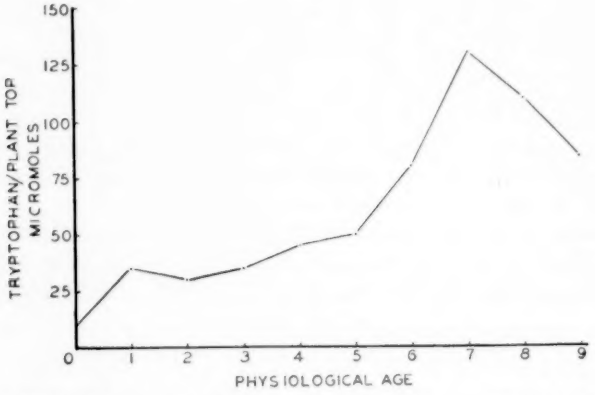

FIG. 3. Tryptophan content of oat plants having the same chronological age but varying in physiological age from the vegetative stage to maturity.

tryptophan in the leaves when compared to those plants in group 0. It is possible that indoleacetic acid was being produced from tryptophan when the plants were held in an unfavorable photoperiod, but this reaction was inhibited markedly when the oat plants were placed in an 16-hour day length. This explanation could account for the marked differences in tryptophan content in the plants of groups 0 and 1.

The glutamic acid content of the plants increased sharply from group 0 up to group 4. Then the amount of glutamic acid in the plant tops decreased about 360 micromoles. After group 6, there was an increase of about 1500 micromoles of glutamic acid in the plant top. The decrease in glutamic acid in the plants from group 4 to group 6 consisted of a loss of about 530 micromoles in the leaves offset by an increase of about 60 micromoles in the stems and 110 micromoles in the fruits. There was an increase in concentration in the roots of 16 micromoles per gram dry weight during this period. While no accurate estimates of the total increase of glutamic acid in the roots was

possible from these experimental data, it is obvious that the oat plants suffered an over-all loss in glutamic acid at this stage of development. It should be pointed out that the amount of proline increased about 330 micromoles in the tops, and proline was also increasing in concentration in the roots at that time. Relations between glutamic acid and proline are well known in other living systems, and it was interesting to note that similar relations seemed to exist in the developing oat plants (fig. 4). The point of interest here is that glutamic acid may serve as a precursor for proline instead of proline being converted into glutamic acid.

KOHLER (9) studied the vitamin content of oats with respect to the stage of maturity and reported that approximately two weeks before the oats headed out there was an increase in folic acid in the plant on the dry-weight basis. While the physiological age of the plants in his work cannot be compared with that of the plants in the present work, it is worthy of note that

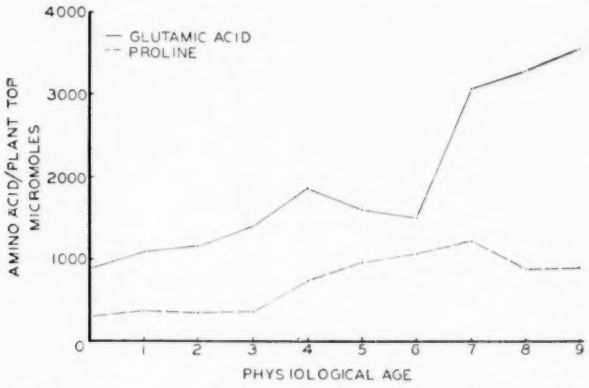

FIG. 4. Glutamic acid and proline content of oat plants. Note the loss of glutamic acid from groups 4 to 6 and the simultaneous increase in proline content.

between the groups 4 and 5 a portion of the loss of glutamic acid could possibly have been converted into folic acid.

There was a loss of serine (ca. 80 micromoles) in the plant top from groups 0 to 3. During this time there was an increase of about 13 micromoles per gram dry weight of this compound in the roots. While some serine might be translocated into the roots at this time, this increase in the roots could not account for the total loss of serine from the plant top. Further, cystine increased about 25 micromoles in the plant top and methionine increased from group 0 to group 1 (ca. 15 micromoles) but decreased in groups 2 and 3 (fig. 5). The demethylation of methionine to form homocysteine and the subsequent condensation with serine to form the unsymmetrical thioether, cystathionine, which cleaves to yield cysteine, has been established by DU VIGNEAUD *et al.* (20). It appears that similar relations occur in developing oat plants.

Generally speaking, the leaves increased in total amino acids from group

0 to 3. This increase was followed by a decrease (groups 3 to 4) when the stems began to elongate and a subsequent increase after flowering. Apparently many of the amino acids were translocated into the stems from the leaves when the stems began rapid elongation, but the heterocyclic and sulphur-containing amino acids did not exhibit this loss between groups 3 and 4. The total amino acids in the stems generally increased as the stems increased in weight. The sixth group exhibited a loss of lysine, proline, glycine, threonine, valine, and leucine in the stems (with respect to group 5) while they contained the same amount of phenylalanine and histidine. These losses in many instances could be accounted for by the presence of these compounds in the fruits.

The fruits showed marked increases in the total amount of amino acids as they matured. In the early stages of fruit development, the amino acids

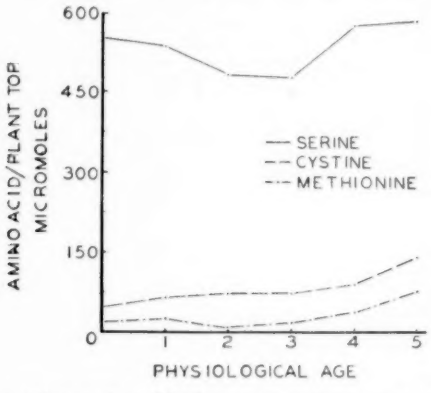

FIG. 5. Serine, cystine, and methionine content of oat plants in the earlier stages of development.

which entered the fruits in the largest amounts were lysine, phenylalanine, glutamic acid, aspartic acid, glycine, valine, leucine, isoleucine, and cystine. Proline was the last amino acid to enter the fruits in an appreciable amount.

It might be well to mention some results in the composition of plants of group 10. It was noted that the development of the plant was retarded somewhat as a result of being continually exposed to a 16-hour day. At the time of harvest, it was noted that exsertion was not complete, and this group corresponded to group 7 on the basis of visual observations. It was interesting to see that the composition of plants of groups 7 and 10 were similar in many instances on a concentration basis.

There are many other relations which can be noted from the experimental evidence presented here, but they would not justify detailed discussion. Some of the more obvious tendencies have been pointed out and these should be emphasized. When the flower primordia were initiated in the oat plant, tryptophan increased in the plant top. It is possible that the trypto-

phan formed in the plant was being converted into indoleacetic acid, and exposure of the plant to a favorable photoperiod inhibited this conversion.

As the stems began to elongate rapidly, many amino acids were translocated from the leaves into the stems. During the shooting stage (groups 0 to 5), the oat plants accumulated a reserve of amino acids in the roots, and after flowering (group 6) these compounds were rapidly translocated into the plant tops. Glutamic acid was translocated into the roots from the tops as development progressed from that of group 4 to that of group 6, but in addition a portion of this compound was apparently lost at this time. It might have been partially converted into proline. In addition, there was evidence that serine was partially converted to cystine.

Summary

Wintok Oats, CI-3424, were grown in eight-hour and 16-hour photoperiods in such a manner that plants of the same chronological age would vary in physiological age from the purely vegetative stage to maturity. The plants were harvested and separated into leaves, roots, stems, and fruits. These were analyzed for fifteen amino acids. Some relations between amino acid composition and physiological age of the plants are discussed.

The authors would like to express their appreciation to the laboratory staff of The Samuel Roberts Noble Foundation for assistance in the routine procedures described in this paper.

LITERATURE CITED

1. Bonner, J. Further experiments on flowering in Xanthium. Bot. Gaz. **110**: 625–627. 1949.
2. Bonner, J. and Thurlow, J. Inhibition of photoperiodic induction in Xanthium by applied auxin. Bot. Gaz. **110**: 613–624. 1949.
3. Bonnett, O. T. The development of the oat panicle. Jour. Agr. Res. **54**: 927–931. 1937.
4. Cardinall, E. V. and Hedrick, L. R. Microbiological assay of corn steep liquor for amino acid content. Jour. Biol. Chem. **172**: 609–612. 1948.
5. Galston, A. W. Indoleacetic-nicotinic acid interactions in the etiolated pea plant. Plant Physiol. **24**: 577–586. 1949.
6. Green, M. and Fuller, H. J. Indole-3-acetic acid and flowering. Science **108**: 415–416. 1948.
7. Hamner, K. C. and Maynard, L. A. Factors influencing the nutritive value of the tomato. U. S. Dept. Agr. Misc. Pub. 502. 1942.
8. Henderson, L. M. and Snell, E. E. A uniform medium for determination of amino acids with various microörganisms. Jour. Biol. Chem. **172**: 15–29. 1948.
9. Kohler, G. O. The effect of stage of growth on the chemistry of grasses. Jour. Biol. Chem. **152**: 215–223. 1944.

10. Leopold, A. C. and Thimann, K. V. The effect of auxin on flower initiation. Amer. Jour. Bot. **36**: 342–347. 1949.
11. Loehwing, W. F. Nutritional factors in plant growth and development. Proc. Iowa Acad. Sci. **49**: 61–112. 1942.
12. Lyson, C. B., Beeson, K. C., and Ellis, G. H. Effects of micro-nutrient deficiencies on growth and vitamin content of tomato. Bot. Gaz. **104**: 495–514. 1943.
13. McCoy, T. A., Bostwick, D. G., and Devich, A. C. Some effects of phosphorus on the development, the B vitamin content, and the inorganic composition of oats. Plant Physiol. **26**: 784–791. 1951.
14. McCoy, T. A. and Wender, S. H. The amino acid requirements of *Streptococcus faecalis* A.T.C.C. No. 6057. Presented at the Seventh Southwestern Meeting of the American Chemical Society, Austin, Texas, December 1951.
15. Maynard, L. A. and Beeson, K. C. Some causes of variation in the vitamin content of plants grown for food. Nutrition Abstracts Revs. **13**: 155–164. 1944.
16. Murneek, A. E. and Whyte, R. O. Vernalization and Photoperiodism. Chronica Botanica Company, Waltham, Massachusetts. 1948.
17. Naylor, A. W. Some effects of growth substances on floral initiation and development in Xanthium. Amer. Jour. Bot. **37**: 681. 1950. (An abstract.)
18. Purvis, O. N. and Gregory, F. G. Studies in the vernalization of cereals. I. A comparative study of the vernalization of winter rye by low temperature and by short days. Ann. Bot. **1**: 569–591. 1937.
19. Sauberlich, H. E. and Bauman, C. A. The effect of dietary protein upon amino acid excretion of rats and mice. Jour. Biol. Chem. **166**: 417–428. 1946.
20. du Vigneaud, V., Kilmer, G. W., Rachele, J. R., and Cohn, M. Mechanism of the conversion in vivo of methionine to cystine. Jour. Biol. Chem. **155**: 645–651. 1944.

ELECTRODE REACTIONS OF ISOLATED CHLOROPLAST FRAGMENTS

H. S. A. GILMOUR, RUFUS LUMRY AND JOHN D. SPIKES

DEPARTMENT OF CHEMISTRY AND DIVISION OF BIOLOGY,
UNIVERSITY OF UTAH, SALT LAKE CITY, UTAH

Received April 17, 1952

In connection with experiments on the lifetime of a postulated light-induced metastable compound in chloroplasts, it became necessary to investigate the effect of illumination on the oxidation-reduction potential of washed chloroplast fragments suspended in a solution free of oxidizing or reducing agents. Potential changes which were observed upon illumination of such systems showed the occurrence of a photo-reduction reaction. This phenomenon is of interest with respect to electrode reactions. The system was free of readily reducible small molecules which indicated that a reaction must have occurred between the metal electrode and the large chloroplast fragments (all of the fragments were sedimented in 10 minutes at 21,000 × g). This reaction appears to be analogous to the Hill reaction in which an added oxidant serves to remove the electrons produced in the oxidation of water to oxygen gas and hydrogen ion by illuminated chloroplast preparations (**5**). The existence of photo-reducible groups in the chloroplast fragments themselves, as described above, may provide an additional approach to the elucidation of the mechanism of the Hill reaction.

Potential changes in the same direction as those reported here have also been observed by MACDOWALL (**3**) who worked with washed chloroplast preparations at low oxygen partial pressures. SPIKES *et al.* (**6**) reported small, essentially irreversible, potential changes towards reduction upon the illumination of washed spinach chloroplasts in an unbuffered sucrose solution in contact with air. FRANCK (**2**) and later TOLMACH (**9**) measured the evolution of oxygen from chloroplasts. The present work describes the oxidation-reduction reactions of washed chloroplast fragments at electrode surfaces.

Materials and methods

Chloroplast fragments of Swiss chard (*Beta vulgaris*, var. Fordhook Giant) were prepared, standardized and stored as previously described for spinach chloroplast preparations (**5**). Spinach (*Spinacia oleracea*) chloroplast fragments were prepared in a similar manner except that they were washed a total of four times by resuspending in 0.5 M sucrose and recentrifuging at 21,000 × g.

Changes in the oxidation-reduction potentials of the chloroplast preparations were measured in special Warburg vessels provided with small calomel and platinum electrodes connected to a Brown Strip Chart Recorder by means of an impedance matching circuit as described elsewhere (**7**). The

reaction systems were 0.1 M in potassium phosphate buffer of pH 6.5, 0.01 M in KCl, and 0.17 M in sucrose. Each reaction system contained a quantity of chloroplast fragments equivalent to 5.1×10^{-7} moles of chlorophyll in the case of chard and 9.1×10^{-7} moles of chlorophyll in the case of spinach unless otherwise specified. Tank nitrogen bubbled through alkaline pyrogallol was passed through the vessels continuously during the course of an experiment. The reaction vessels were shaken in a Warburg bath at 15° C and were illuminated with incandescent light (20,000 lux) or with incandescent light filtered through a gelatin filter which cut out wave lengths shorter than 5800 Å (8,000 lux). The results obtained were independent of the color of light used.

Results and discussions

The nature and magnitude of the potential changes which result when washed chloroplast fragments are illuminated in the absence of added oxidizing and reducing agents are shown in figure 1. These changes do not

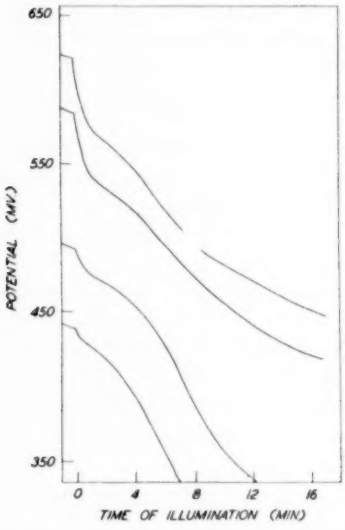

FIG. 1. Curves from Brown Recorder showing photoreduction of washed spinach chloroplast fragments suspended in phosphate buffer and sucrose solutions. Chloroplast fragment concentration equivalent to 9.3×10^{-7} moles of chlorophyll. Potassium phosphate buffer concentration 0.1 M, pH 6.5. Sucrose concentration 0.17 M. Potassium chloride concentration 0.01 M.

primarily result from photogalvanic effects, since the potentials do not return to their original values when the illumination is stopped (**4**). According to the convention used in this paper, the potential of a reduction-oxidation half-cell is increased if the equilibrium shifts in favor of the oxidized form.

Experiments with coated electrodes

As the control of the electrode potential by such large particles as the microscopically visible chloroplast fragments seemed surprising, experiments were performed in which the platinum electrode was covered with a collodion coating which could be penetrated by small molecules, but not by the large chloroplast fragments. Figure 2 shows the potential changes recorded

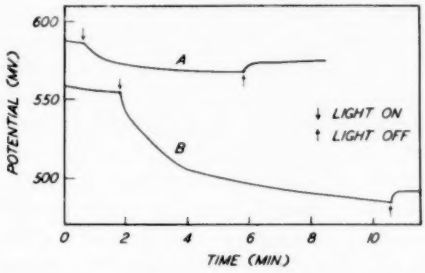

Fig. 2. Brown Recorder trace of potential changes measured by a collodion-coated electrode; A. in a solution of buffered chloroplast fragments; B. after the addition of potassium ferricyanide. Chloroplast fragment concentration equivalent to 8×10^{-5} moles chlorophyll. Sucrose concentration 0.17 M. Potassium chloride concentration 0.01 M.

in the same system with and without the addition of potassium ferricyanide when a collodion-coated platinum electrode was used. These curves show, apart from a small photogalvanic effect, that this electrode responds to illumination only if small carrier molecules, such as ferricyanide are present. It is apparent then, that the potential change in the absence of such small molecules is due to a shift in the relative concentrations of oxidized and reduced forms of unknown constituents of the fragments, located at or near the surface of these particles. Chard chloroplast fragments showed photoreduction curves similar to those of spinach preparations.

Photoreduction experiments

The individual potential *versus* time plots (fig. 1) resemble in part the familiar electrometric titration curve. Normally the inflection point of such a plot corresponds to the standard half-cell potential of an oxidant, and therefore occurs at the same potential from one experiment to another. However, in figure 1 the inflection point potential varies by more than 100 mv. for different experiments. As the standard half-cell potential of the natural oxidant was therefore uncertain, these curves did not lend themselves to interpretation by the method of Spikes *et al.* (**6**).

The progressive decrease in slope of the lower parts of the potential *versus* time curves (fig. 1) may be due to back-oxidation by oxygen produced during the course of the reaction. This assumption is supported by the fact that the reaction will not proceed unless the system is continuously swept out with purified nitrogen. Photo-inactivation or heat-inactivation

cannot be an important factor here, for the addition of potassium ferricyanide to illuminated chloroplast preparations which had been photo-reduced resulted in a rapid photoreduction of the ferricyanide.

Pre-oxidation experiments

In an attempt to interpret the unusual results presented in the preceding section, chloroplast systems were oxidized prior to illumination with some oxidant which added no new molecular or ionic species to see if the initial steep part of the photoreduction curve could be separated from the region where the inflection point occurred. However, none of the agents used was capable of performing the necessary oxidation without inactivating the chloroplast fragments. Molecular oxygen had no effect. Hydrogen peroxide oxidized the system and the excess peroxide could be removed with either manganese dioxide or platinum black. However, both methods left the chloroplasts insensitive to illumination. No better success was realized by electrolytic oxidation with a large anode and small cathode (**8**). After such an oxidation, the system was unstable and drifted rapidly towards reduction.

Back-titration of photoreduced fragments

The nature of the unknown chloroplast oxidant reacting at the electrode was of interest. To determine the amount of oxidant present, light-reduced chloroplast fragments were back-titrated with several oxidants. Because the potential of these suspensions was increased upon addition of acid, and was unstable in the acid region, reduced fragments were titrated in a basic medium where the potential was more stable and the oxidizing agent finally

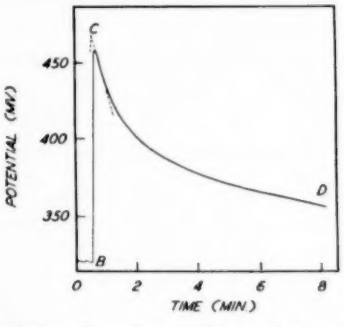

Fig. 3. Curve from Brown Recorder showing typical potential changes observed during an oxidative titration of light-reduced chard chloroplast fragments, buffered at pH 8. Point B, 0.6 ml. of 0.00015 M potassium ferricyanide plus 0.4 ml. phosphate buffer added. Point C, extrapolated final value of potential after completion of the initial rapid reaction. Curve CD, disappearance of potassium ferricyanide in a slow reaction. Chloroplast fragment concentration equivalent to 5.1×10^{-7} moles of chlorophyll. Sucrose concentration 0.17 M. Potassium chloride concentration 0.01 M. Potassium phosphate buffer concentration 0.1 M.

chosen was relatively stronger. As photoreduction was slow in basic solutions, it was necessary to photoreduce the fragments at a pH of 6.5, and then adjust the pH to 8 before titration.

Various oxidizing agents were tried, including sodium dichromate, potassium permanganate, *p*-benzoquinone and potassium ferricyanide. Only the latter was considered suitable, and even when using it the back-oxidation was not a simple process. As can be seen in figure 3, on the addition of a small amount of ferricyanide the potential changed rapidly in the direction of oxidation and then moved more slowly towards reduction. The possibility that the shape of the curve BCD (fig. 3) might simply be due to a slow reaction between the added ferricyanide and the photoreduced unknown oxidant in the chloroplasts was considered, but such a mechanism could not explain the relation between the length of BC (fig. 3) and the amount of ferricyanide added since this relation was such that the data could be used to construct a typical potentiometric titration curve by the method of FIESER (**1**).

The second possible interpretation of figure 3 was that the following reaction sequence was operative:

(1) $$X_r + \text{ferricyanide} \rightleftarrows X_o' + \text{ferrocyanide}$$

(2) $$X_o' \xrightarrow{k} Y$$

where X_r is the photoreduced molecule, X_o' is an unstable oxidized product, and Y is its decay product. If reaction 1 is assumed to be rapid and reversible, and reaction 2 to be slow and irreversible, the disappearance of X_o' in a first-order decay reaction would shift the equilibrium of reaction 1 to the right, thus decreasing the ratio of ferricyanide to ferrocyanide. As this couple appeared to control the electrode, the net result would be a steady drift towards lower potentials. However, the observation that for many of the curves the same amount of ferricyanide is reduced between two given times militates against both these hypotheses.

A more satisfactory hypothesis is that the rapid initial rise of the curve from B to C (fig. 3) results from the rapid reaction of ferricyanide with that part of the photoreduced chloroplast material located on the surface of the fragments, while the gradual fall in the curve from C to D results from the reduction of ferricyanide by photoreduced material from the inside of the fragments, according to the following reaction scheme in which the asterisk indicates a photoexcited molecule, X is the unknown chloroplast redox substance, and the subscripts have the following meanings: r, reduced form; o, oxidized form; s, molecules on the surface of the chloroplast fragments; and i, molecules in the interior of the chloroplast fragments. As the concentration of X_{os} is kept practically constant at its maximum value by reaction 5, reaction 6, and consequently the rate of reduction of potassium ferricyanide, becomes effectively first order in X_{ri}.

(3) $\text{chlorophyll} \rightarrow \text{chlorophyll}^*$

(4) $\text{chlorophyll}^* + X_o \rightarrow X_r + \text{chlorophyll}$

(5) $X_{rs} + \text{ferricyanide} \rightleftarrows X_{os} + \text{ferrocyanide}$

(6) $X_{ri} + X_{os} \underset{\leftarrow}{\overset{k}{\rightleftarrows}} X_{oi} + X_{rs}$

Reaction 5 in which the equilibrium between ferricyanide and X_{rs} is established rapidly will be considered first by using the type of construction shown in figure 3 to determine by extrapolation the potential at point C (fig. 3), which is assumed to be the final potential for reaction 5. The data from seven experiments performed on photoreduced spinach fragments have been plotted in figure 4 to show the relation between the amount of ferricyanide added and the potential difference between points corresponding to B and C in figure 3. As the inflection point in this titration curve was approximately the E^o value for the ferrocyanide-ferricyanide couple (6) it appears that at pH 8, the E^o of the unknown substance was below the potential to which the system was photoreduced. However, these data could still be used to determine the normality of the unknown substance, for the volume of added oxidant which would have been necessary to drive this substance from, say 90% reduced to 10% reduced when passing through the E^o of the unknown substance, is just the value that was needed to drive the ferricyanide concentration from 90% reduced to 10% reduced when passing through the ferricyanide E^o.

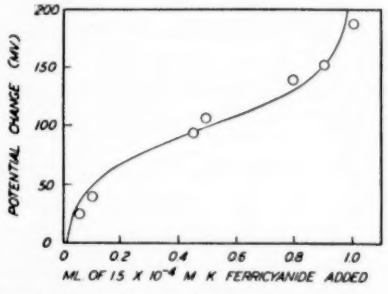

FIG. 4. Potentiometric discontinuous titration of a rapidly re-oxidizable substance in photoreduced spinach chloroplast fragments by the method of FIESER (1). Circles represent experimental points; solid line represents a theoretical titration curve for a one-electron change. Chloroplast fragment concentration equivalent to 9.3×10^{-7} moles chlorophyll. Sucrose concentration 0.17 M. Potassium chloride concentration 0.01 M. Potassium phosphate buffer concentration 0.1 M.

Data from titrations of the less thoroughly washed chard chloroplast suspensions were used to calculate the unknown oxidant concentration for this material. As it was not practicable in this case to reduce each sample to the same potential before adding the potassium ferricyanide, the calculations became slightly more involved. In order to interpret the data ob-

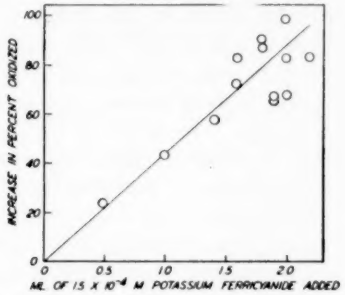

FIG. 5. Potentiometric discontinuous titration of photoreduced chard chloroplast fragments, assuming a one-electron change, and an E^o of 0.442 mv. Chloroplast fragment concentration equivalent to 5.1×10^{-7} moles of chlorophyll. Potassium phosphate buffer concentration 0.1 M. Sucrose concentration 0.17 M. Potassium chloride concentration 0.01 M.

tained from the titration, it was necessary to assume a value for E^o, and for the number of electrons involved per molecule oxidized. For these assumptions, one could plot the change in per cent. reduced *versus* the amount of oxidant added for various runs, as in figure 5. By repeating the calculation using different values for E^o, and for both one- and two-electron changes, the E^o giving the minimum average deviation of the points from the best straight line was found. For chard, the ferricyanide E^o of 0.442 mv. and a one-electron change gave the smallest deviation. Although this method of interpretation does not permit a determination of the exact shape of the titration curve, as with the spinach data, the average deviation proved to be very sensitive to small changes in the E^o assumed. Even for the best E^o

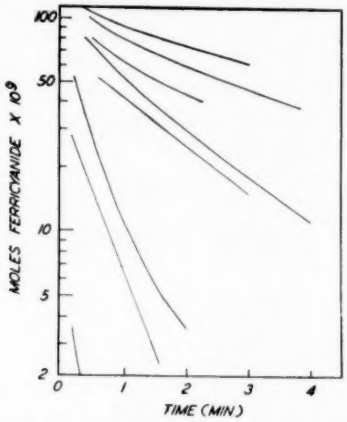

FIG. 6. Reaction of potassium ferricyanide with a slowly oxidizable substance in photoreduced chard chloroplast fragments. Data obtained from curves of the type CD of figure 3. Different amounts of potassium ferricyanide were added to the photoreduced fragments for each of the curves shown. Potassium ferricyanide added at zero time.

chosen, however, the average deviation was 12%. Chard fragments were extremely oxygen-sensitive in their photoreduced state, and it is likely that this fact contributed to the large experimental error. However, if our assumptions are correct, the calculations gave an approximate value for the normality of X_s.

Consider now reaction 6, which proceeds at a slower rate. Figure 6, which is a plot of data from curves of the type CD (fig. 3), shows (except for the three lowest curves) that essentially the same amount of ferricyanide is reduced between two given times for every experiment. One can therefore find an average value for the amount of ferricyanide consumed in a given time interval. If the reaction in question is indeed a first order oxidation of X_{ri}, then it can be shown that

$$X_1 = \frac{(\Delta \text{ferrocyanide})_1^2}{2(\Delta \text{ferrocyanide})_1 - (\Delta \text{ferrocyanide})_2} \tag{7}$$

where X_1 is the amount of X_{ri} present at time t_1, $(\Delta \text{ferrocyanide})_1$ is the amount of ferricyanide reduced in the time interval from t_1 to $t_1 + \Delta t$, and $(\Delta \text{ferrocyanide})_2$ is the amount of ferricyanide reduced in the time interval from t_1 to $t_1 + 2\Delta t$. It will be noticed that the three lower curves (fig. 6) give slower rates of reduction of ferricyanide. For this reason, calculations were performed with data from the top five curves only.

Once the value of X_1 was determined, the first-order rate constant could be found from the equation:

$$k = \frac{2.3}{2\Delta t} \log_{10} \frac{X_1}{X_1 - (\Delta \text{ferrocyanide})_2} \tag{8}$$

From a knowlege of X_1 and k, the amount of X_{ri} present at the time of addition of ferricyanide (X_{ri}^o) could be found, since $X_{ri}^o = X_1 e^{kt_1}$. In this manner, a value of 0.68 min.$^{-1}$ was found for the rate constant, and the concentration of X_{ri}^o was calculated to be 0.17 equivalents per mole of chlorophyll. The fundamental data obtained through application of these equations are summarized in table I.

TABLE I

FUNDAMENTAL CHARACTERISTICS OF CHLOROPLAST FRAGMENTS AS CALCULATED.

	Spinach	Chard
Equivalents X_s per mole chlorophyll	0.14	0.67
Equivalents X_{ri}^o per mole chlorophyll formed during 40 minutes of photo-reduction	.....	0.17
First order reaction rate constant (k)	.....	0.68 min.$^{-1}$
Rate of reduction of potassium ferricyanide (Hill reaction) expressed as moles ferricyanide/(mole chlorophyll-min.)	0.33	2.38

From the data in table I, it can be shown that the average rate of formation of X_{ri}^{o} was about 0.2% of the Hill reaction rate. It is interesting to compare this value with the observations of other workers. FRANCK (**2**) measured the rate of oxygen evolution from illuminated chloroplasts as a function of time, and reported an initial rate which was about 2% that of the Hill reaction. This value decreased in about four minutes to 0.3%. The rate of malate formation found by VISHNIAC and OCHOA (**10**) is comparable to our rate of formation of X_{ri}^{o}.

On the basis of the preceding discussion, the hypothesized situation during the photoreduction of chloroplast fragments may now be visualized as follows. Two or more naturally occurring redox materials are involved in the photoreduction, but only one of the materials may react at the electrode. The variation of the apparent E^{o} (fig. 1) from one experiment to another can now be explained as due to a non-equilibrium situation; for while the molecules X_s can react at the electrode, they cannot establish rapid equilibrium with X_i. The kinetics of such a situation is complicated, and with the data presently available no definite confirmation of this suggestion is possible.

It is not, however, too early to suggest that the photoreduction mechanism involved in these observations is the same as that in the Hill reaction. Light produces reduced substances which can be titrated with typical Hill-reaction oxidants. Furthermore, unpublished results demonstrate that the reducible substances can be alternately reduced and oxidized many times, by alternate exposure to light and then to oxygen. Oxygen is a convenient oxidant in that it is readily removed from the system by equilibration with nitrogen. It is probable that the groups reacting at the electrode are the direct chemical species which react with the Hill oxidant. In this connection it is interesting to note that the reducible substance is probably part of a large water-insoluble molecule. It would be useful to find the standard half-cell potential of the unknown and to determine whether it is located only at the surface or throughout the chloroplast fragments. On the one hand the presence of a secondary and more slowly reducible substance may indicate a storage place for reducing power. If so, this substance is not reduced in the presence of Hill-reagent oxidants since its rate of reduction is slow compared with that of such oxidants. On the other hand the slow material may be some substance located on the surface of the chloroplast fragments which becomes reducible only when chloroplasts are fragmented. In the latter case however, the secondary substances cannot explain the general failure to note reduction potentials of the expected order for carbon dioxide reduction in Hill reaction systems, for whole chloroplasts exhibit this same limitation.

Summary

Isolated chloroplast fragments from Swiss chard and from spinach can react reversibly at a platinum electrode. This reaction is not due to small water-soluble molecules.

A photoreduction of the isolated chloroplast fragments occurs in oxygen-free suspensions which may be followed potentiometrically.

Discontinuous oxidative titration of the photoreduced chloroplast fragments leads to the conclusion that two substances capable of slowly reacting with each other are involved in the photoreduction, but that only one of these substances reacts at the electrode.

The significance of the rapidly reduced substance as the possible intermediate locus of reducing power in the Hill reaction is discussed.

This work was supported by grants from the United States Atomic Energy Commission and the University of Utah Research Fund.

LITERATURE CITED

1. Fieser, L. F. The potentials of some unstable oxidation reduction systems. Jour. Amer. Chem. Soc. **52**: 4915–4940. 1930.
2. Franck, J. Photosynthetic activity of isolated chloroplasts. Rev. Mod. Phys. **17**: 112–119. 1945.
3. MacDowall, F. D. H. Private communication. 1951.
4. Rabinowitch, E. I. Photosynthesis and Related Processes. I. P. 76. Interscience Publishers, New York. 1945.
5. Spikes, J. D. Stoichiometry of the photolysis of water by illuminated chloroplast fragments. Arch. Biochem. Biophys. **35**: 101–109. 1952.
6. Spikes, J. D., Lumry, R., Eyring, H., and Wayrynen, R. E. Potential changes in suspensions of chloroplasts on illumination. Arch. Biochem. **28**: 48–67. 1950.
7. Spikes, J. D., Lumry, R., Rieske, J. S., and Marcus, R. J. Methods for measuring and recording oxidation-reduction potentials in plant preparations. (In preparation.)
8. Swann, S., Jr. Electrolytic reactions. In: Technique of Organic Chemistry, Vol. II. Pp. 199–201, A. Weissberger, Ed. Interscience Publishers, New York. 1948.
9. Tolmach, L. J. The influence of triphosphopyridine nucleotide and other physiological substances upon oxygen evolution from illuminated chloroplasts. Arch. Biochem. Biophys. **33**: 120–142. 1951.
10. Vishniac, W. and Ochoa, S. Photochemical reduction of pyridine nucleotides by spinach grana and coupled carbon dioxide fixation. Nature **167**: 768–769. 1951.

EFFECT OF INORGANIC IONS AND THEIR CONDUCTANCES ON GEOTROPIC CURVATURE OF THE AVENA COLEOPTILE

A. R. SCHRANK

Department of Zoology, University of Texas, Austin, Texas

Received May 1, 1952

Introduction

Experimental results of Wilks and Lund (**4**) show that filling isolated Avena coleoptiles taken from seedlings 34 to 36 mm. long with Shive's solution reduces the longitudinal electrical polarity from a maximum of 60 to less than 10 millivolts. When the solution is removed from the inside of the sheath, the original electrical polarity promptly reappears. These observations were presented as proof of internal shunting of some of the polar units which contribute to the longitudinal electrical polarity of the isolated coleoptile. Previously reported data also show that geotropic curvature of the Avena coleoptile is inhibited by filling the internal cylinder with an electrolyte such as Shive's solution (**3**). The degree of inhibition is directly dependent on the concentration and specific conductance of the solution used. If it could be demonstrated that such inhibition of curvature is due exclusively to the shunting effect of the electrolyte, then it would appear that an inherent electrical field is a prerequisite for growth curvature responses.

In the present paper data are presented to show the effects of solutions of various monovalent cations (Li, Na, K) on geotropic curvature in an attempt to distinguish between the specific effects of concentration and conductance. These ions were selected because equal concentrations of LiCl, NaCl, and KCl should have different shunting effects depending on their equivalent conductances.

Materials and method

Etiolated seedlings of *Avena sativa*, Victory Strain (U. S. Department of Agriculture C.I. 2020) were grown and prepared as indicated in previous publications (**2, 3**). The coleoptiles, which were used in these experiments, were isolated from intact seedlings that were 30 ± 1 mm. long, perfectly straight, and had a well developed root system. A cut was made all the way around the coleoptile about 5 mm. apical to the seed so that the leaves could be pulled out of the basal end. The coleoptile cylinders were filled with one of the various solutions by the use of a hypodermic syringe with a needle tapered to fit snugly into the basal end of the sheath. This technique made it possible to force the solution slowly into the entire length of the coleoptile without excessive mechanical stimulation since only the most basal cells came in contact with the needle. After filling, the coleoptiles were placed in glass holders such as shown in the inset of figure 1 and were permitted to

remain undisturbed in the vertical position for one hour to recover from any mechanical stimulation of handling. At the end of this time they were placed in the horizontal position for the indicated duration. Geotropic curvatures were measured by following the vertical tip movement with an ocular micrometer or by determining the angular curvature at the end of the experiment. Angular curvatures were measured by the method of JUDKINS (**1**) or by making shadowgraphs which were projected, and the angles were measured with a protractor.

Electrical conductances of the solutions were measured in the standard way using a Kohlrausch Slide Wire (Leeds and Northrup number 4258) arrangement and the typical conductivity cell. All conductance determinations were made at 25° C and all experiments were performed at the same temperature.

Experimental results

Curve I in figure 1 presents typical data on the upward curvature of coleoptiles (average of ten) filled with air and placed in holders containing Shive's solution. This curve may, therefore, be regarded as a control or base line for those which follow. The average angular curvature after 60 minutes in the horizontal position was 35 degrees. Curves II, III, and IV

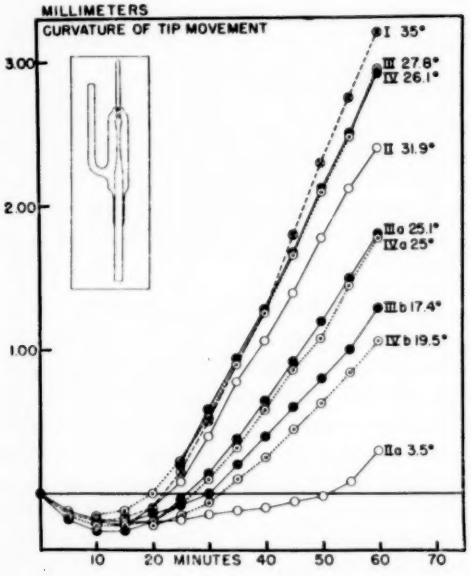

FIG. 1. Upward curvatures of isolated Avena coleoptiles which were placed in the horizontal position for 60 minutes. Each curve represents average values of 10 or more coleoptiles. Angular curvatures are shown at the end of each curve. Internal cylinders were filled with the indicated solutions. Curve I, air; curve II, 0.01 *N* LiCl; curve III, 0.01 *N* NaCl; curve IV, 0.01 *N* KCl; curve IIa, 0.05 *N* LiCl; curve IIIa, 0.05 *N* NaCl; curve IVa, 0.05 *N* KCl; curve IIIb, 0.1 *N* NaCl; curve IVb, 0.1 *N* KCl.

show the geotropic response of coleoptiles that were filled with 0.01 normal solutions of LiCl, NaCl, and KCl respectively. The values of the specific conductances given in this paper are more accurate than the values given for concentrations. No extraordinary precautions were taken in preparing the stock solutions and the various dilutions, but the conductivity determinations were verified to be accurate to within $\pm$ 1%.

It is noted from curves III and IV and the corresponding angular curvatures that 0.01 normal NaCl and KCl solutions both inhibit geotropic curvature, but the degree of inhibition of the two solutions is practically identical which means that the extent of inhibition is the same for solutions with specific conductances of 0.0011 and 0.00132. Curve II, from coleoptiles filled with 0.01 normal LiCl solution with a specific conductance of 0.000944, presumably indicates that LiCl inhibits geotropic curvature more effectively than either NaCl or KCl of the same concentration. When the comparison is made on the angular basis, which is the more reliable method of measuring actual curvature in experiments of this kind, the LiCl solution produces less inhibition than either the NaCl or KCl. (31.9° for LiCl compared to 27.8° and 26.1°.) This difference in the two methods of measurement appears because the zone of curvature of coleoptiles that were filled with the LiCl solution is closer to the apex. Under such conditions the apex moves a shorter distance for a given angular curvature. This fact must always be kept in mind when interpreting data of this kind.

In the next series of experiments the coleoptiles were filled with 0.05 normal solutions of the same salts. The results are given by curves IIa, IIIa, and IVa. In general, these curves and the corresponding angular curvatures show that the zone of curvature is kept closer to the apex by this concentration than by the previous one. This observation is interpreted to indicate that the location of the initial bending region is to some extent dependent on concentration and specific conductance as well as the nature of the salt used. Curves IIIa (NaCl) and IVa (KCl) are again practically identical. The specific conductances of the NaCl and KCl solutions were 0.00564 and 0.00706 respectively. It is relevant to note that 0.05 normal NaCl and KCl solutions give about the same average angular curvature as 0.01 normal solutions, even though there is about a fourfold difference in the specific conductances. Coleoptiles filled with 0.05 normal LiCl solution with a specific conductance of 0.0046 (curve IIa) show only very slight geotropic bending which indicates that LiCl has extensive effects on curvature responses which are not directly dependent on the specific conductance of the solution.

Curves IIIb and IVb and corresponding angular curvatures show the inhibitory effects of filling the coleoptiles with 0.1 normal solutions of NaCl and KCl with specific conductances of 0.0106 and 0.0128, respectively (Handbook of Chemistry and Physics). These solutions have a more pronounced inhibitory effect than the next more dilute, but the differences between the curves IIIb and IVb and the corresponding angular curvatures are not considered significant.

TABLE I

GEOTROPIC CURVATURE OF ISOLATED AVENA COLEOTILES AS AFFECTED BY SPECIFIC CONDUCTANCES OF 0.01 *N* SOLUTIONS OF LiCl, NaCl AND KCl.

Solution in holder	Specific conductance at 25° C	Contents of coleoptile	Number of cases	Average degrees curvature
				90 minutes
Distilled water		air	55	52.9
LiCl	0.00094	air	41	48.5
NaCl	0.0011	air	41	50.3
KCl	0.00132	air	38	49.9
LiCl	0.00094	LiCl	36	38.3
NaCl	0.0011	NaCl	37	54.0
KCl	0.00132	KCl	37	48.6
				60 minutes
LiCl	0.00094	LiCl	60	28.7
NaCl	0.0011	NaCl	58	26.0
KCl	0.00132	KCl	60	26.5

The data in table I are presented to analyze further the possible relationship of specific conductance to curvature. The first four lines in the table show the effects of the various salts in the holders only while the coleoptiles were filled with air. In these experiments the plants were left in the horizontal position for 90 minutes and only angular measurements were made using the shadowgraph technique. It was unnecessary to measure the apex displacement in this series of experiments because the average distance between the curved region and the apex is independent of the salt used when the plants are left in the horizontal position for 90 minutes. When the holders are filled with distilled water and the cylinders are filled with air, the average geotropic curvature obtained is 52.9°. Filling the holders with solutions of LiCl, NaCl, and KCl with the indicated conductances causes slight inhibition of upward curvature. When both the holders and coleoptiles are filled as noted in the table, LiCl effectively inhibits geotropism. The inhibitory effect of this concentration occurs largely during the last 30 minutes in the horizontal position rather than during the first 60 minutes. Comparison may be made with the angular curvatures corresponding to curves II, III, and IV in figure 1. Since the conductance of the LiCl solution is less than that of NaCl or KCl solutions, it is further apparent that the inhibitory effect of LiCl is due to properties other than the specific conductance of the solution. The data in the last three lines of table I were obtained from a series of experiments which duplicate those represented by curves II, III, and IV in figure 1.

Comparison of the angular curvatures attained after 60 and 90 minutes in the horizontal position when the coleoptiles were filled with 0.01 normal NaCl solution reveals another interesting phenomenon. Data in figure 1 and table I show that after 60 minutes in the horizontal position, geotropic curvature of coleoptiles filled with 0.01 normal NaCl solution is significantly inhibited. When the plants are left in the horizontal position for 90 minutes,

the inhibitory effect of the NaCl disappears and the coleoptiles attain the same magnitude of curvature manifested by the controls. Similarly, there appears to be at least a partial recovery from curvature inhibition due to 0.01 normal KCl during the last 30 minutes in the horizontal position.

Figure 2 presents summary curves in which average degrees of curvature obtained in 60 minutes are plotted against the specific conductances of the various solutions used. It is at once apparent that as the conductance of each solution is increased, the magnitude of geotropic curvature inhibition also becomes greater, but the increase of inhibition caused by increasing the NaCl and KCl solutions from 0.01 to 0.05 normal is very slight. The increased effects of higher conductances are generally obtained only with corresponding increases in solution concentrations. The points on curves II, III, and IV, which correspond to a concentration of 0.01 normal, appear to

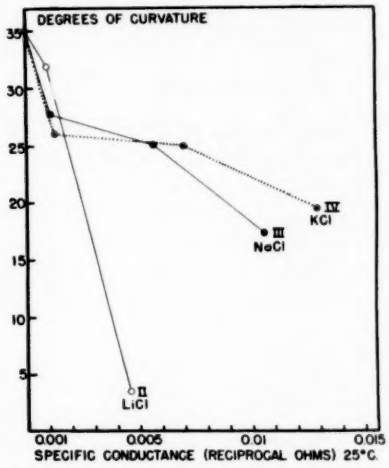

FIG. 2. Inhibition of geotropic curvature of isolated Avena coleoptiles as related to specific conductances of various concentrations of solutions of LiCl (curve II), NaCl (curve III), and KCl (curve IV) with which the sheaths were filled.

indicate a direct relationship between the exclusive property of specific conductance and the magnitude of curvature inhibition. Actually, however, the difference between 27.8 and 26.1 degrees (NaCl and KCl) is not significant as determined by the t test so the indicated relationship is not valid. Curve II shows the inhibitory effects of LiCl which are also dependent on specific conductance and concentration. Comparison of this curve to curves III and IV further emphasizes the special LiCl effect which was previously noted.

Discussion

In a previous paper (**3**) is was demonstrated that inhibition of geotropic curvature is dependent on the concentration and specific conductance, but is not dependent on the osmotic pressure of the solution with which the coleoptiles were filled. Glycerol and various dilutions of Shive's solution were used in the former experiments. The data in the present paper are in general

agreement with the previous results. When the solution concentrations of LiCl, NaCl, and KCl are increased from 0.01 normal to 0.1 normal, the magnitude of inhibition of geotropic curvature is extended for each of the salts. The specific conductance of these solutions also increases with concentration.

In figure 2 the inhibition of geotropic curvature is related to the exclusive property of specific conductance. The magnitude of inhibition appears not to be a function of the selected characteristic of specific conductance of the various concentrations of the solutions that were used to fill the coleoptiles. The conductance effects apparently are masked by different types of responses to the individual electrolytes that were used.

A striking phenomenon observed in these experiments is the extensive inhibitory effect of the LiCl solutions, which also is not a function of the exclusive property of specific conductance. The possible implications of this observation are not apparent at present, but it would appear worthwhile, in this connection, to investigate the effects of LiCl on the bioelectrical properties of the Avena coleoptile.

Summary

Experiments were performed to test the effects of various concentrations and specific conductances of LiCl, NaCl, and KCl on the geotropic curvature of the Avena coleoptile.

The magnitude of curvature inhibition is enhanced as the solution concentration and specific conductance of a given salt are increased, but the extent of curvature inhibition is not directly dependent on the exclusive property of specific conductance of solutions of LiCl, NaCl, and KCl from 0.01 to 0.1 normal.

Except for the 0.01 *N* solution, when the coleoptiles were stimulated for 60 minutes, LiCl proved to be the most effective inhibitor of geotropic curvature. This effect appears to be due to a property of LiCl other than its specific conductance.

This project was supported by the University of Texas Research Institute. The Avena seeds were generously supplied by the U. S. Department of Agriculture.

LITERATURE CITED

1. Judkins, W. P. A device for measuring Avena coleoptile curvature. Science **101**: 568. 1945.
2. Schrank, A. R. Relation between electrical and curvature responses in the Avena coleoptile to mechanical stimuli. Plant Physiol. **19**: 198–211. 1944.
3. Schrank, A. R. Inhibition of curvature responses by shunting the inherent electrical field. Plant Physiol. **25**: 583–593. 1950.
4. Wilks, S. S. and Lund, E. J. The electric correlation field and its variation in the coleoptile of *Avena sativa*. Bioelectric Fields and Growth, pp. 24–75. University of Texas Press, Austin. 1947.

FILTERS FOR THE ISOLATION OF NARROW REGIONS IN THE VISIBLE AND NEAR-VISIBLE SPECTRUM[1]

ROBERT B. WITHROW AND LEONARD PRICE

DIVISION OF RADIATION AND ORGANISMS, ASTROPHYSICAL OBSERVATORY, SMITHSONIAN INSTITUTION, WASHINGTON, D. C.

Received August 22, 1952

Introduction

Investigations of photochemical reactions in higher plants and other biological systems frequently require the irradiation of large areas with well-defined regions in the visible and near-visible spectrum. Large glass filters or dyed gelatin filters of the Wratten type are costly. Inexpensive theatrical gelatin filters, dyed cellophane, or other dyed plastics are available, but these are limited in variety of spectral characteristics and few of them have sharp absorption bands.

The laboratory preparation of large dyed gelatin films is very inexpensive. These films are particularly useful for studies in plant photochemistry because the transmission bands can be adjusted to meet the special requirements of each problem. On the basis of laboratory experience and of various reports in the literature (**6, 7, 10, 11, 13**), the following information is presented on the technique of preparation of dyed gelatin films and the composition and transmission data for a few typical filters. In addition, data are presented on the transmission of water and solutions of copper sulphate and ferrous ammonium sulphate, which are useful as primary filters for the removal of the infrared and certain other regions of the visible and near-ultraviolet spectrum.

With water or aqueous solutions as primary filters and dyed gelatin films as secondary filters, it frequently is possible to isolate single lines of the mercury arc spectrum and fairly narrow regions from incandescent lamp sources. Since the gelatin films can be prepared conveniently in sizes up to at least one square meter, relatively high irradiances can be produced over areas of a square meter or more, making it possible to grow mature plants with spectral regions that are well defined and sufficiently narrow for many physiological studies.

The dyed gelatin films to be described were prepared by the general method of casting hot solutions of dye and gelatin onto plate glass. After the layer has gelled, it is allowed to dry. If the glass has been treated previously with silicone, the film can be stripped from the casting plate and mounted between panes of window glass.

[1] Published by permission of the Secretary of the Smithsonian Institution.

Materials and methods

Preparation of dyed gelatin filters

The stock gelatin solution is prepared from any high-grade, clarified, edible gelatin yielding a clear solution free of excessive opalescence. The composition for about 1300 ml. of solution is gelatin, 150 gm.; sorbitol, 50 gm.; sodium benzoate, 1 gm.; and water, 1000 ml. The sorbitol serves as a plasticizer and keeps sufficient water in the film to prevent brittleness and cracking. It is superior to glycerin or ethylene glycol under conditions of varying humidity. Excess sorbitol produces soft films, especially at high humidity. The sodium benzoate serves as a fungicide. The sorbitol and sodium benzoate are dissolved in the water; then the gelatin is added and the whole is heated in a water bath to about 60° C. It is important that the gelatin solution not be overheated as this causes yellowing and appreciable absorption in the near-ultraviolet. A foam is formed on the surface but gradually disappears if the container is kept covered during heating. Any remaining bubbles may be removed with a slip of glass or a piece of filter paper but a more effective method is to use an antifoaming agent. A few milliliters of N butyl alcohol applied to the surface will cause most of the bubbles to break. A silicone product, Antifoam A (Dow Corning Corporation, Midland, Michigan), is very active when applied as a 0.5% solution in benzene or ethyl ether or in a mixture of 25% benzene and 75% butyl alcohol. When using the solvent mixture, it is necessary to dissolve the Antifoam in the benzene first and then add the butyl alcohol. A few milliliters of this solution may be added to the dye-gelatin solution at almost any stage where bubbles or foam are problems.

The gelatin filters are specified in terms of the amount of dye in a unit area of a standard film of gelatin. The formulas of tables I and II give the grams of dye per 1000 cm.^{2} of surface when using 100 ml. of stock gelatin solution containing the requisite amount of dye. Only water-soluble dyes are suitable for use in gelatin films requiring sharp transmission bands. The three most useful classes of dyes are the basic, acid, and substantive or direct cotton dyes. The biological stains as a class are useful but lack the variety which is available in commercial fabric dyes. Often biological stains are available in a relatively inexpensive form as fabric dyes under various trade names.

The Society of Dyers and Colourists (**12**) presents material which contains information on the chemical structure and properties of the older commercial dyes and the various names which have been used for each. Conn (**2**) gives similar information on those dyes used as biological stains. The various older and newer editions published by the American Association of Textile Chemists and Colorists (**1**) are especially useful as references to those dyes of American manufacture available during the year of issue.

The dye solution is prepared by dissolving the required amount of dye in hot distilled water of a volume equal to that of the stock gelatin solution

to be used. Acid or alkali is added to prevent precipitation of the gelatin, resulting in cloudy films. About 0.3 ml. of ammonium hydroxide per gram of acid dye or 0.3 ml. of glacial acetic acid per gram of basic dye usually is adequate. In general, acid and basic dyes, when mixed in the same film, produce cloudy films. There are some exceptions, however.

Many dyes are variable mixtures of a homologous series, together with colorless inorganic diluents. Consequently, different lots from the same manufacturer may not yield the same results. It always is necessary that each lot of dye be analyzed with a spectrophotometer and the proportions adjusted to give the desired filters. For the testing of new lots of dye or of new dye combinations, it is convenient to cast the films on lantern slide cover glasses.

The casting of films should be done on plate glass of about 6 mm. thickness for sizes up to 40 cm. square. For larger sizes, 9 to 12 mm. thicknesses should be used for sufficient rigidity. The following details are given for 9 mm. plate glass, 64 cm. square. Such a casting plate yields trimmed filters 60 cm. × 60 cm. The casting plate must be mounted on another smaller piece (ca. 60 × 60 cm.) of plate glass, with thin felt or blotting paper between them to give uniform support and to prevent sagging. The mounting plate is first carefully leveled and raised slightly off the bench top by using thin slips of glass or shimming brass. The casting plate is cleaned with alcohol, and a narrow strip of masking tape is applied to the edges so as to cover from 1 to 2 cm. of the outer edge of the top surface. The remaining inner surface is then coated with a thin layer of a silicone fluid (**5, 8**) containing unhydrolyzed methyl chloro-silanes which hydrolyze on exposure to a humid atmosphere, with the release of hydrochloric acid, to yield a silicone. A 5% solution of Dry Film SC-87 in benzene (General Electric Company), or Beckman Desicote, is very satisfactory for this purpose. Silicone oils or grease (**11**) have not proved nearly so effective in preventing the adhesion of the gelatin film to the glass.

The silane solution is applied with a cotton swab and all the excess polished off with a cloth. It is important that the masked edge remain untreated in order that the gelatin film in drying may adhere at the edge and not shrink. The vertical edges of the plate also should be treated with silane as this helps to prevent the hot solution from flowing over the edge of the glass. If it starts to run over, surface tension causes it to break readily. After the application of the silane, the masking tape is removed and a band of thinned gelatin solution or glue is painted on the edge. This band is not absolutely essential, but materially helps in holding the edges of the gelatin film to the edges of the casting plate during drying.

The solutions of dye and gelatin are heated separately to about 60° C in a beaker, mixed, and the surface bubbles removed. The casting plate should be warmed slightly. A bank of nine 75-watt internal reflector lamps mounted on a suitable panel is satisfactory for warming the casting plate. The mixture then is poured gently down a large glass rod onto the center of the plate

so as to prevent splashing and the formation of more bubbles. The liquid will have to be teased to the edges of the plate with a glass rod or a piece of thin glass since the silicone surface tends to make the solution pull into the center of the casting plate. When the mixture touches the outer untreated edge, it adheres and will not pull away while drying. If the silicone has accidentally reached the edge of the glass and the solution does not adhere, alcohol can be used to remove some of the silicone, or gentle rubbing with a glass rod will frequently cause adhesion.

The films are dried in a dust-free room of low humidity with gentle circulation of air. Violent air movement before gelation will disturb the surface. Usually the films will dry within 8 to 12 hours, after which they can be stripped from the plate by loosening from the edge or cutting them out with a razor blade. The films should be stored in a dry environment

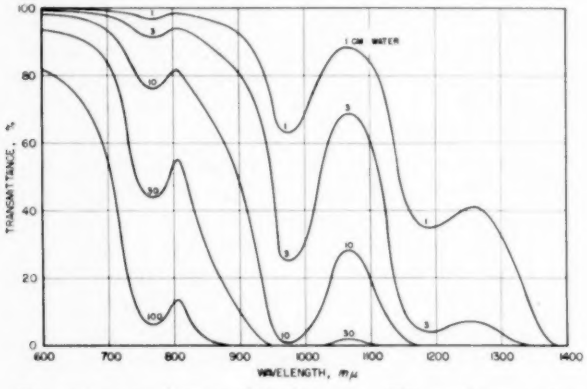

FIG. 1. Per cent. transmittance of liquid water. The numbers on the curves refer to centimeters of path length.

between layers of paper. If the relative humidity is high and the films tend to absorb moisture, a thin film of a clear plastic coating, such as Krylon, may be applied from a pressurized dispenser. After stripping, the films can be mounted between panes of window glass treated on the inner surface with a silane to prevent adhesion of the filter. The films should be cut exactly equal to the size of the glass to be used and mounted with masking tape to prevent light leakage around the edges of the assembly.

Results and discussion

PRIMARY FILTERS

Organic dyes gradually fade to leuco-compounds on intense irradiation, and dyed gelatin films deteriorate rapidly at temperatures above 50° C. In addition, all organic dyes transmit freely in the near-infrared; most of the blue and green dyes absorb little beyond 680 to 720 mμ. A few dyes such as naphthol green B can be used in high densities to absorb the red and near-

infrared up to 800 or even 900 mμ, but such infrared absorption is rare. Therefore, it usually is necessary to use a primary filter of water or solutions of copper sulphate or ferrous ammonium sulphate to remove the near-infrared and most of the radiated energy of the lamp source, especially for incandescent sources.

The spectral transmittance data for liquid water are given in figure 1. These curves were plotted from the original tables of CURCIO and PETTY (**3**), who very kindly supplied the authors with their laboratory data. Since water is a single component system and is being compared to air, the values are per cent. transmittance as a function of wave length, in accordance with the recommended nomenclature (**4**). Primary filters have been used in various thicknesses of from 1 to 30 cm. A 1-cm. layer of water alone absorbs

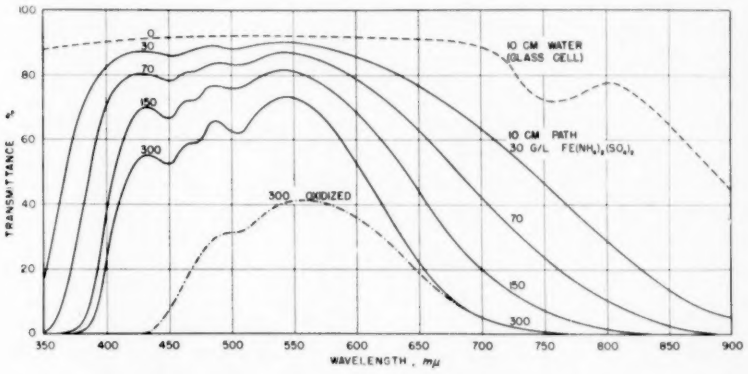

FIG. 2. Per cent. transmittance of various concentrations of ferrous ammonium sulphate in a path length of 10 centimeters. The solutions were prepared in 2% sulphuric acid. The concentrations were 30, 70, 150, and 300 grams per liter of solution as indicated on the solid curves. The upper dashed curve is for water; the lower dash-dot curve is for 300 gm./l. solution exposed to the air for two weeks.

nearly all the energy beyond 1400 mμ. For a 10-cm. layer, the cut-off is about 1150 mμ and at 30 cm. about 950 mμ. For most large filter applications, a 10-cm. thickness of primary filter is a good compromise and is relatively easy to cool and maintain, especially in open tanks.

The per cent. transmittances (including water absorption balanced against air) of a path length of 10 cm. for various concentrations of ferrous ammonium sulphate in 2.0% sulphuric acid (**9**) are given in figure 2. The per cent. transmittancies (balanced against water) of a path length of 10 cm. for various concentrations of copper sulphate in 0.5% sulphuric acid are shown in figure 3. All concentrations are in grams per liter of solution. The copper sulphate data in figure 3 are presented as transmittancy and do not include water absorption. Therefore, the copper sulphate data can be extrapolated to other concentrations and thicknesses by the application of the Bouguer-Beer law. For cells of 10 cm. or less, water absorption may be

neglected between 350 and 680 mμ, with an error of less than 5%. These data were obtained with reagent chemicals using a Beckman spectrophotometer calibrated against solution standards (4).

Since ferrous ammonium sulphate oxidizes rapidly to the ferric form in open cells exposed to the air, it is necessary to retard the rate of oxidation by adding a small amount of clean iron wire to the acidulated solution. In figure 2 are given transmission data of a fresh solution of ferrous ammonium sulphate at 300 gm./l. and the same solution after exposure to the air for two weeks. There is little change in the red end of the transmission band, but oxidation seriously attenuates the shorter wave lengths. When iron wire is added, the rate of deterioration is retarded very markedly. Ferrous ammonium sulphate is especially useful in conjunction with long wave pass

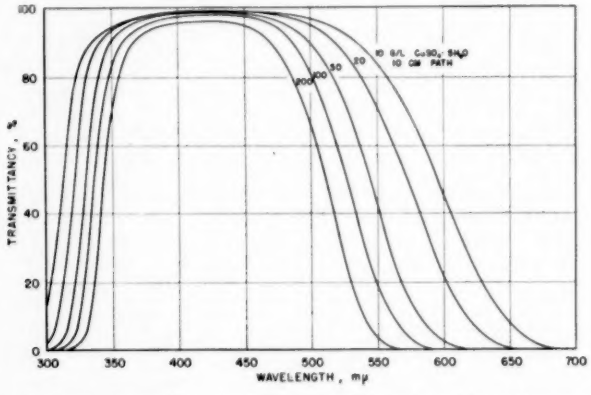

FIG. 3. Per cent. transmittancy of copper sulphate in a 10-centimeter path length at the concentrations indicated in grams per liter of solution made up in 0.5% sulphuric acid.

gelatin films for isolating narrow red bands in the region of 650 to 700 mμ. Copper sulphate is more practical for shorter wave lengths and may be used up to 700 mμ if it is possible to tolerate low transmission maxima.

COMPLETE FILTERS

In table I and figure 4 are given the composition and transmission data for a few long wave pass secondary gelatin filters. These filters may be used for narrowing the transmission bands of other filters. Those filters with limits in the red are especially useful when combined with copper sulphate and ferrous ammonium sulphate solutions. It will be noted that the red long wave pass filters usually involve a blue or green dye combined with an orange or yellow dye, the latter to block out the blue or green transmission bands of the former.

In table II and figure 5 are presented the composition and per cent. transmittance of a few band pass filters. These values are for the complete

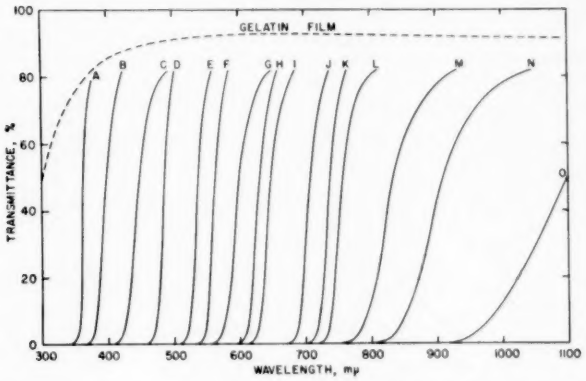

FIG. 4. Transmittance of long wave pass dyed gelatin filters of the composition given in table I.

combination of primary and secondary filters. Reflection losses are not included, however.

For highly critical work where, for example, a photochemical reaction is being excited in a region of low effectiveness, as would be the case at the limits of the action spectrum, the possible presence of film irregularities, such as pinholes or low density areas, becomes serious. In order to minimize the effect of such irregularities the filter may be cast in two or three films with each film having a half or a third of the total dye and gelatin. Sufficient additional water is added to allow the solution to flow over the casting plate. This system is useful also for very high density filters in which one film cannot carry the full complement of dye without clouding.

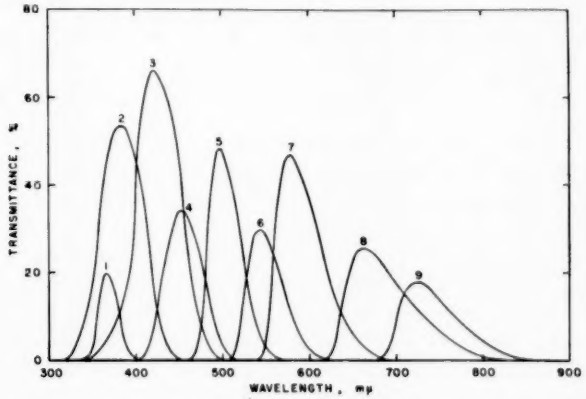

FIG. 5. Transmittance of complete band pass filter systems of composition given in table II.

TABLE I

COMPOSITION OF LONG WAVE LENGTH PASS FILTERS.

Symbol in figure 4	Short wave length limit	Secondary filter of dyed gelatin film	
		Density	Name of dye
	$m\mu$	*gm./1000 cm.²*	
A	350	1.0	2-Naphthol-6,8-disulphonic acid, sodium salt (G salt)
B	375	0.5	Esculin*
C	420	2.0	8-Hydroxyquinoline sulphate
D	470	0.5	Picric acid*
E	520	2.0	Tartrazine
F	545	1.0	Tartrazine
		1.0	Orange G
G	565	0.5	Chrysoidine Y
H	605	0.7	Pontamine fast red 8 BL
I	620	2.0	Pontamine fast red 8 BL
J	680	0.3	Pontacyl green BL extra conc. 200%
		0.7	Pontamine fast red 8 BL
K	705	0.4	Acid green No. 1854 New**
		0.5	Pontamine fast red 8 BL
L	725	1.0	Acid green No. 1854 New†
		0.5	Pontamine fast red 8 BL
M	765	1.5	Pontamine fast blue 4 GL conc. 150%
		0.5	Pontamine fast red 8 BL
N	820	3.0	Pontamine diazo blue NA conc. 200%
O	930	1.5	Naphthol green B extra conc. 125%
		0.5	Pontamine fast red 8 BL

*Esculin and picric acid are dissolved in 95% alcohol prior to mixing with gelatin stock solution.

**Acid green no. 1854 New was especially prepared by DuPont; Pontacyl brilliant blue E (1.5 gm./1000 cm.²) may be substituted in filter K.

†Pontamine sky blue FF extra conc. 150% (1.5 gm./1000 cm.²) may be substituted in filter L for Acid green no. 1854 New.

Neutral filters

When working with very low irradiances, it is frequently necessary to attenuate the source intensity by a precisely known factor. Small reductions may be obtained by the use of panes of diffusing glass or woven wire cloth. For large factors of 10 or more, neutral filters are more practical. These filters are prepared by adding graphite suspensions such as aquadag or India ink to the gelatin solution. Such filters transmit uniformly throughout the visible and near-ultraviolet spectrum with slightly decreasing transmittance with decreasing wave length in accordance with the laws of small particle scattering. Such neutral filters have little skewing effect on the transmission bands when used in combination with other filters.

TABLE II

COMPOSITION OF BAND PASS FILTER SYSTEMS.

Symbol in figure 5	Wave length limits	Primary filter 10 cm. aqueous		Secondary filter of dyed gelatin film	
		Concentration	Salt*	Density	Name of dye
	$m\mu$	$gm./l.$		$gm/1000\ cm.^2$	
1	340–395	100	$CuSO_4 \cdot 5H_2O$	0.5	New Fuchsin (1 cm. aqueous soln.)**
2	325–445	100	$CuSO_4 \cdot 5H_2O$	0.15	New Fuchsin
3	345–490	100	$CuSO_4 \cdot 5H_2O$	0.05	Victoria pure blue BO
				0.01	Crystal violet extra pure APNX
4	410–510	50	$CuSO_4 \cdot 5H_2O$	0.2	Victoria pure blue BO
				1.5	8-Hydroxyquinoline sulphate
5	465–560	200	$CuSO_4 \cdot 5H_2O$	0.5	Picric acid
6	510–620	20	$CuSO_4 \cdot 5H_2O$	0.4	Naphthol green B extra conc. 125%
				1.0	Tartrazine
7	545–690	10	$CuSO_4 \cdot 5H_2O$	1.0	Tartrazine
				1.0	Orange G
8	620–800	150	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	2.0	Pontamine fast red 8 BL
9	685–850	100	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.3	Pontacyl green BL extra conc. 200%
				0.7	Pontamine fast red 8 BL

*All primary filters were prepared in a solution of 0.5% sulphuric acid.
**This filter has not been prepared satisfactorily in gelatin.

Summary

The preparation of large dyed gelatin filters 60 $cm.^2$ is described. Details are given for the preparation of dyed gelatin solutions, the casting of the solutions onto plate glass treated with silicones, stripping of the films from the glass, and mounting. Transmission curves are presented for typical gelatin filters and for various concentrations of solutions of copper sulphate and ferrous ammonium sulphate as primary infrared filters.

The authors wish to express their indebtedness to Dr. H. M. Benedict, who spectrophotometrically analyzed many of the hundreds of dyes which

have been examined, and to Mr. E. Horowitz, who suggested the use of iron wire for stabilizing the ferrous ammonium sulphate solutions.

LITERATURE CITED

1. American Association of Textile Chemists and Colorists. Technical Manual and Yearbook. Howes Publishing Co., Inc., New York.
2. Conn, H. J. Biological Stains. Biotech Publications, Geneva, New York. 1946.
3. Curcio, J. and Petty, C. C. The near infrared absorption spectrum of liquid water. Jour. Opt. Soc. Amer. **41**: 302–304. 1951.
4. Gibson, K. S. Spectrophotometry. Nat. Bur. Standards Circ. 484. 1949.
5. Gilbert, P. T., Jr. Silicone water-repellents for general use in the laboratory. Science **114**: 637–640. 1951.
6. Jones, L. A. Light filters for the isolation of narrow spectral regions. Phot. Jour. **70**: 337–346. 1930.
7. King, E. J. and Ventura, S. Light filters of coloured "Perspex." Nature **168**: 702. 1951.
8. Norton, F. J. Organo-silicon films. Gen. Elec. Rev. **47**: 6–16. 1944.
9. Pfund, H. H. Screens for the near infrared. Jour. Opt. Soc. Amer. **29**: 56–58. 1939.
10. Shenk, J. H., Hodge, E. S., Morris, R. J., Vickett, E. E., and Brode, W. R. Plastic filters for the visible and near infrared regions. Jour. Opt. Soc. Amer. **36**: 569–575. 1946.
11. Smith, B. Methods for making filters transmitting the near ultraviolet and absorbing visible light. Science **104**: 490–491. 1946.
12. Society of Dyers and Colourists. Colour Index. F. M. Rowe, Editor. Bradford, England. 1924.
13. Withrow, R. B. and Benedict, H. M. Dyed gelatin light filters. Trans. Ill. Eng. Soc. **31**: 69–81. 1936.

PHOSPHOGLUCONIC DEHYDROGENASE IN HIGHER PLANTS

ROBERT C. BARNETT,[1] HELEN A. STAFFORD, ERIC E. CONN[2] AND BIRGIT VENNESLAND

DEPARTMENT OF BIOCHEMISTRY AND DEPARTMENT OF BOTANY, UNIVERSITY OF CHICAGO, CHICAGO, ILLINOIS

Received November 3, 1952

A dehydrogenase catalyzing the oxidation of glucose-6-phosphate by triphosphopyridine nucleotide (TPN) has been shown to be widely distributed in higher plants (**2, 5**). This finding suggested that plants might form pentose by a TPN-dependent, two-step oxidation sequence similar to that already described in other organisms (**6, 8, 17**) and shown in equations 1 and 2.

(1) glucose-6-phosphate + TPN^+ + H_2O $\longrightarrow$ 6-phosphogluconate + TPNH + $2\,H^+$

(2) 6-phosphogluconate + TPN^+ $\longrightarrow$ pentose phosphate + CO_2 + TPNH

This paper presents evidence that enzyme systems catalyzing reaction (**2**) are widespread in higher plants. After this work was started, evidence for the presence of such enzymes in peas and spinach was presented by AXELROD and BANDURSKI (**3**).

Materials and methods

The barium salt of 6-phosphogluconate was prepared according to the method of SEEGMILLER and HORECKER (**18**). Solutions were prepared for use by dissolving the material in HCl, removing the barium with potassium sulphate, and adjusting the pH to 7.4. Various samples of barium phosphogluconate contained 1.7 to 2.0 μM of organic phosphorus per mg. and 0.01 to 0.03 μM of inorganic phosphorus per mg. The fructose content, according to the method of ROE (**16**), was not more than 0.03 μM per mg., and the glucose-6-phosphate content, according to assay with glucose-6-phosphate dehydrogenase (**11**) was 0.08 to 0.13 μM per mg. of the barium salt. The phosphogluconate content was calculated from the organic phosphorus content, after correction for the impurities. Ribose-5-phosphate was prepared by acid hydrolysis of adenylic acid or adenosine triphosphate according to the procedure described by LEPAGE and UMBREIT (**12**) and ALBAUM and UMBREIT (**1**). A preparation of TPN^+ of 70% purity was generously supplied by Dr. M. A. Mitz of Armour Laboratories. Oxidized glutathione (GSSG) was purchased from Schwartz or was prepared by oxidizing GSH with H_2O_2 as previously described (**5**). Tris (trishydroxymethyl amino-

[1] Fellow of the American Cancer Society, Inc. (recommended by the Committee on Growth of the National Research Council). Present address: Department of Physiology, University of Texas, Galveston, Texas.

[2] Present address: Division of Plant Nutrition, University of California, Berkeley, California.

methane) was purchased from Commercial Solvents Corporation and recrystallized from an acetone-water mixture. Pentose was determined according to the method of MEJBAUM (**13**). The change in optical density was measured at 660 mμ, after heating in a boiling water bath for 25 minutes. Standards were made from D-ribose.

Except for the case of wheat germ and turnip root, the enzyme preparations were made by grinding the plant, with sand if necessary, but without the addition of water. Leaves were ground in the frozen state; fruits and roots were peeled and ground in a meat grinder. The coarse solids were strained off through cheesecloth and the smaller particulate matter was removed by centrifugation in the cold at 22,000 × g. All extracts were dialyzed against 0.025 M phosphate buffer (pH 7.4) for 24 hours at 3° C. Turnip root was peeled and ground in a meat grinder. The pulp was frozen with an equal volume of water, thawed, and squeezed through cheesecloth. Centrifugation and dialysis were then carried out as before. Dialyzed water extracts of wheat germ prepared as previously described (**5**) gave a high value in the orcinol test for pentose which was a disadvantage when accurate determinations of pentose formation were desired. A fractionation procedure was devised which gave a protein preparation which was rich in enzymes, but contained relatively little nucleic acid. This wheat germ protein fraction will be referred to as the WP preparation.

Wheat germ was extracted for 30 minutes at room temperature with four volumes of distilled water. The extract was strained through cheesecloth and centrifuged at 20,000 × g. for 20 minutes at 4° C. All subsequent operations were carried out in a cold room at this temperature. The supernatant was treated with $MnCl_2$ to remove nucleic acid, by a modification of the method described by KAUFMAN *et al.* (**10**). For each liter of extract, 20.4 ml. of 1.0 M $MnCl_2$ were added slowly with stirring. The solution, which had a final pH of about 5.5, was allowed to stand overnight. The heavy precipiate which formed was centrifuged off at 3,000 × g. The supernatant was stored at – 15° C for 48 hours, thawed rapidly and centrifuged again at 20,000 × g. for 20 minutes to remove additional precipitate which had appeared. The pH was adjusted to 7 with concentrated NH_4OH, and 250 gm. of solid $(NH_4)_2SO_4$ were added slowly for each liter of solution. Mechanical stirring was used during these operations, and the pH was kept at 7 by further addition of NH_4OH. After 24 hours, the bulky precipitate was centrifuged down and discarded. To each liter of supernatant, 75 gm. of $(NH_4)_2SO_4$ were then added with stirring. The precipitate was centrifuged down and dissolved in a minimal volume of 0.025 M phosphate buffer, pH 7.4, and dialyzed with stirring against the same buffer to remove the $(NH_4)_2SO_4$. The final volume was about 260 ml. of protein solution for every kilogram of wheat germ originally employed. The original extract contained about 30 mg. dry weight per milliliter after dialysis. Measurement of the ratio of the optical densities at 280 and 260 mμ indicated that about 13% of this material was nucleic acid (**19**). The amount of nucleic acid removed by the $MnCl_2$ treatment varied in different preparations, but

it was always possible by repetition of the procedure to bring the nucleic acid content down to about 1% or less. The final dry weight of this WP preparation was about 40 mg./ml.

Results

Demonstration of phosphogluconic dehydrogenase

All of the plant preparations contained the enzyme glutathione reductase (**2, 5**), which catalyzes the reaction shown in equation 3.

(3) $$GSSG + TPNH + H^+ \longrightarrow 2\,GSH + TPN^+$$

This system may be coupled with any reaction in which TPN is reduced, such as the reactions shown in equations 1 and 2. The presence of phosphogluconic dehydrogenase, therefore, could be easily detected by demonstrating that GSSG was reduced when phosphogluconate, GSSG, and a catalytic amount of TPN were incubated with the plant preparation to be tested. Table I contains representative results obtained with enzyme preparations

TABLE I

Reduction of glutathione by 6-phosphogluconate.

Source	Amount of extract	Time	Phosphogluconate added	GSSG reduced	
				Complete system	Phosphogluconate omitted
	ml.	*min.*	*μmoles*	*μmoles*	
Wheat germ	0.5	45	3.7	2.7	0.2
Cantaloupe fruit	1.6	30	10.0	3.1	0.2
Parsley leaf	1.0	30	10.0	7.6	0.1
Spinach leaf	1.0	30	5.0	3.2	0.3
Parsnip root	1.0	30	5.0	1.1	0.0
Cucumber fruit	1.6	45	10.0	7.3	0.1
Turnip root	1.6	60	10.0	2.9	0.3

made from seven different sources. Enzyme and phosphogluconate were incubated at 30° C in 3.7×10^{-2} M tris buffer of pH 7.4 with 9 μM GSSG and 30 γ TPN; total volume, 2.7 ml. The reaction was stopped by the addition of 0.3 ml. 20% HPO_3, the precipitate was centrifuged down, and an aliquot of the supernatant was titrated with iodate as previously described (**2, 5**). The enzyme preparation of wheat germ was the dialyzed water extract. The enzyme preparation of cucumber fruit consisted of the dialyzed juice concentrated to one third of the original volume by drying in the frozen state. Controls were always run in which TPN, GSSG, and phosphogluconate were omitted separately. The first two controls were uniformly low and therefore have not been included. The third control without substrate is given because it sometimes reaches an appreciable value.

No negative results were obtained in these experiments. Considerable quantitative variations were found, however, even with different preparations made from the same source. The figures must not be regarded as a quantitative measure of the amount of phosphogluconic dehydrogenase present in the plant, except in the sense that they set a minimal value (**2**).

Phosphogluconic dehydrogenase was also demonstrated spectrophotometrically by measurement of the increase in light absorption at 340 mμ when enzyme and substrate were incubated with TPN. The results confirmed the conclusions reached on the basis of the titration procedure. The specificity of the enzyme for pyridine nucleotide was likewise tested spectrophotometrically. No evidence for any reduction of diphosphopyridine nucleotide by phosphogluconate was ever found, even with enzyme mixtures that caused an almost immediate reduction of 0.2 μM of TPN.

Pentose and CO_2 production from phosphogluconate

In another series of experiments, analyses were made for pentose and CO_2 formed during the reduction of GSSG by phosphogluconate. Representative results of these experiments are shown in table II.

TABLE II

COMPARISON OF GLUTATHIONE REDUCTION WITH FORMATION OF PENTOSE AND CO_2.

Source	Amount of extract	Time	GSSG reduced	CO_2 formed	Pentose formed
	ml.	*min.*	*μmoles*	*μmoles*	*μmoles*
Wheat germ (WP)	0.3	45	8.6	6.0	5.6
Cantaloupe fruit	1.0	60	1.8	1.7	1.3
Parsley leaf	0.7	10	6.0	3.9	2.1
	0.7	20	6.9	5.5	1.6
	0.7	40	8.7	6.0	1.6
Spinach leaf	0.7	15	1.7	1.7	1.4
	0.7	45	2.7	1.9	2.2
	0.7	90	3.8	2.1	1.7
Cucumber fruit	1.6	15	4.0	2.3	3.8
	1.6	30	7.0	6.4	6.7
	1.6	60	9.1	7.4	4.8
Turnip root	1.6	20	1.8	0.6	0.4
	1.6	40	2.8	1.5	1.4
	1.6	60	3.2	1.6	1.6

The reactions were carried out in double arm Warburg vessels in an atmosphere of N_2, with yellow phosphorus in the center well. Reaction mixtures were identical with those described for table I, except that 10 μM of phosphogluconate were used in all cases. After incubation, 0.3 ml. of 20% HPO_3 was tipped in, and the CO_2 formed during the reaction was calculated by the standard procedure, with corrections for CO_2 initially present. The contents of the vessels were centrifuged, and aliquots of the supernatants were used for pentose and GSH determinations. The results recorded in table II have been corrected for the small amounts of GSSG reduction, and pentose and CO_2 formation which occurred in the absence of added phosphogluconate. With most of the sources studied, the course of the reaction was followed with time, and a number of these experiments have been included.

In all instances where a given enzyme preparation catalyzed a reduction

of GSSG by phosphogluconate, CO_2 and pentose were found to be reaction products, but the amounts of CO_2 and pentose formed were always smaller than the amount of GSSG reduced. The above discrepancies might be accounted for both by the formation of pentoses with orcinol spectra different from that of ribose, and by the further metabolism of the pentose. The orcinol spectra of the reaction products was, therefore, examined. The parsley leaf preparation and the WP fraction from wheat germ were selected for these studies, since preliminary experiments indicated that they give quite different results. With parsley, an absorption maximum at 660 mμ was predominant at 10 minutes, but in succeeding time intervals, the spectrum shifted to give a peak at about 580 mμ. The absorption maximum at 660

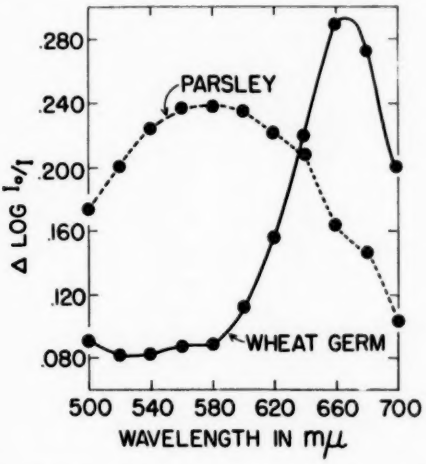

FIG. 1. The absorption curve shown for parsley was determined on a filtrate from a mixture set up as in table II for a 40-minute incubation period. The one for wheat germ was obtained from a mixture comparable to that in table II except that 0.5 ml. enzyme was used and the incubation time was 30 minutes. Absorption spectra were obtained from mixtures incubated with and without phosphogluconate. The curves represent the differences between these spectra.

mμ is characteristic of pentoses, and the seven-carbon sugar, sedoheptulose, has been reported to give a maximum at 600 mμ (**9**), whereas hexoses give absorption at shorter wave lengths. The orcinol spectra obtained with parsley indicated, therefore, that a pentose product formed initially was further metabolized, possibly in part to sedoheptulose. With wheat germ, on the other hand, there was a steady rise of the band at 660 mμ with no decline at later time intervals. The pentose which was formed was apparently not further metabolized. Figure 1 shows the characteristic difference in the orcinol spectra finally observed with the enzymes from the two sources.

Positive results were obtained when the qualitative color test specific for the seven-carbon ketose (**4, 14**) was applied to the incubation mixture with parsley leaf enzyme, but not with wheat germ. The spectral changes

of the orcinol test and the results of the heptulose color test suggested that the parsley leaf preparation contained an enzyme system metabolizing pentose in a manner similar to that described by HORECKER and SMYRNIOTIS (**9**). Such a system appeared to be absent in the wheat germ preparation. The action of the enzymes on ribose-5-phosphate was further investigated in order to obtain confirmation of these conclusions.

RIBOSE-5-PHOSPHATE BREAKDOWN

No action of the wheat germ WP preparation on ribose-5-phosphate could be detected. When ribose-5-phosphate was incubated with the parsley leaf enzyme, however, pentose disappeared, as shown by the decrease in the orcinol absorption band at 660 mμ. Furthermore, a reduction of GSSG occurred when TPN and GSSG were incubated with ribose-5-phosphate in the presence of the enzyme. For example, when 5 μM of ribose-5-phosphate were incubated with one ml. of enzyme for 30 minutes, 3.5 μM of pentose disappeared and 1.6 μM of GSSG were reduced. The oxidoreduction was not required for pentose breakdown, since pentose disappearance was almost as great in the absence of GSSG and TPN as in their presence. The decrease in the 660 mμ absorption band was accompanied by an increase in absorption at about 580 mμ. At the same time the qualitative heptulose color test became positive. These results confirmed the experiments done with phosphogluconate, and showed further that TPN could be reduced by pentose phosphate or by some product derived from it. A parsley leaf preparation in which glucose-6-phosphate dehydrogenase had been completely inactivated was still able to cause a reduction of GSSG in the presence of ribose-5-phosphate and TPN. This observation ruled out the possibility that the reduction of GSSG observed with ribose-5-phosphate might be due to an indirect formation of glucose-6-phosphate from pentose phosphate (**15**). SABLE (**17**) found that yeast preparations formed triose phosphate from ribose phosphate; and HORECKER and SMYRNIOTIS (**9**) found that when sedoheptulose phosphate was formed from pentose phosphate with a liver enzyme preparation, triose phosphate also appeared. The possibility that the reduction of GSSG in the TPN-ribose-5-phosphate system might be due to a TPN-triose-phosphate dehydrogenase was therefore entertained. Such an enzyme has been found by GIBBS (**7**) in pea leaves. However, the coupled reduction of GSSG by ribose-5-phosphate in the presence of the parsley leaf enzyme was completely uninfluenced by arsenate. A similar coupled reduction of GSSG independent of arsenate occurs with fructose disphosphate in the presence of the enzyme of parsley leaf and will be further described in a subsequent paper.

Discussion

The wide distribution of phosphogluconic dehydrogenase and glucose-6-phosphate dehydrogenase in higher plants suggests that this oxidative degradation of hexose occupies a significant position in plant carbohydrate metabolism. The problem of the further metabolism of pentose in these sources has been sketchily investigated in the experiments described in this

paper, and the presence of enzyme systems metabolizing pentose was proved only for the case of the parsley leaf. The data in table II show, however, that with both spinach leaf and cucumber fruit, an initial rise in pentose formation from phosphogluconate was followed by a decrease on further incubation. This may be regarded as indirect evidence that these tissues likewise metabolize pentose. AXELROD and BANDURSKI (private communication) have demonstrated pentose disappearance and sedoheptulose formation with spinach leaf enzyme.

The discrepancies in the stoichiometry shown in the results of table II could be partially explained by further metabolism of pentose. Such an explanation, however, could not account for the data obtained with wheat germ. The failure to demonstrate pentose breakdown in the case of the wheat germ preparation may have been due to inactivation of the enzymes during the procedure employed to lower the pentose content of extracts from this source.

Summary

A phosphogluconic dehydrogenase dependent on TPN has been demonstrated in wheat germ, cantaloupe and cucumber fruits, parsley and spinach leaves, and parsnip and turnip roots. Since all plant sources tested contained glutathione reductase, the oxidation of phosphogluconate was coupled with the reduction of GSSG, and pentose orcinol-reacting material and CO_2 were shown to be formed as products of such reactions. The stoichiometry indicated the occurrence of other reactions in addition to the oxidation of phosphogluconate to pentose and CO_2. A preparation from parsley leaves caused a disappearance of ribose-5-phosphate, with an appearance of the qualitative test for seven-carbon ketoses, and an unidentified TPN dependent oxidative step.

This project was aided in part by a grant from the American Cancer Society on recommendation by the Committee on Growth of the National Research Council and by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. The authors are indebted to General Mills, Inc., Minneapolis, Minnesota, for a generous supply of S-50 wheat germ.

LITERATURE CITED

1. ALBAUM, H. G. and UMBREIT, W. W. Differentiation between ribose-3-phosphate and ribose-5-phosphate by means of the orcinol-pentose reaction. Jour. Biol. Chem. **167**: 369–376. 1947.
2. ANDERSON, D. G., STAFFORD, H. A., CONN, E. E., and VENNESLAND, B. The distribution in higher plants of triphosphopyridine nucleotide-linked enzyme systems capable of reducing glutathione. Plant Physiol. **27**: 675–684. 1952.
3. AXELROD, B. and BANDURSKI, A. S. Oxidative metabolism of hexose phosphates by higher plants. Fed. Proc. **11**: 182. 1952.

4. Bevenue, A. and Williams, K. T. Further evidence indicating the specificity of the orcinol spray reagent for keto heptoses on paper chromatograms. Arch. Biochem. Biophys. **34**: 225–227. 1951.
5. Conn, E. E. and Vennesland, B. Glutathione reductase of wheat germ. Jour. Biol. Chem. **192**: 17–28. 1951.
6. Dickens, F. and Glock, G. E. Direct oxidation of glucose-6-phosphate, 6-phosphogluconate and pentose-5-phosphate by enzyme of animal origin. Biochem. Jour. **50**: 81–95. 1951.
7. Gibbs, M. Triosephosphate dehydrogenase and glucose-6-phosphate dehydrogenase in the pea plant. Nature **170**: 164–165. 1952.
8. Horecker, B. L., Racker, E., Cohen, S. S., Lipmann, F., Lampen, J. O., Schlenk, F., and Dische, Z. The metabolism of pentose and triose phosphates. In: A Symposium on Phosphorus Metabolism, W. D. McElroy and B. Glass, Editors. Johns Hopkins Press, Baltimore, Maryland. 1951.
9. Horecker, B. L. and Smyrniotis, P. The enzymatic formation of sedoheptulose phosphate from pentose phosphate. Jour. Amer. Chem. Soc. **74**: 2123. 1952.
10. Kaufman, S., Korkes, S., and del Campillo, A. Biosynthesis of dicarboxylic acids by carbon dioxide fixation. Further studies of the "malic" enzyme of *Lactobacillus arabinosus*. Jour. Biol. Chem. **192**: 301–312. 1951.
11. Kornberg, A. Enzymatic synthesis of triphosphopyridine nucleotide. Jour. Biol. Chem. **182**: 805–813. 1950.
12. LePage, G. A. and Umbreit, W. W. The occurrence of adenosine-3-phosphate in autotrophic bacteria. Jour. Biol. Chem. **148**: 255–260. 1943.
13. Mejbaum, W. Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. Zeit. physiol. Chem. **258**: 117–120. 1937.
14. Nordal, A. and Klevstrand, R. Studies of the constituents of Crassulacean plants. I. Paper chromatographic investigations of the free sugars of some Sedum, Sempervirens, and Crassula species. Acta Chem. Scand. **5**: 85–88. 1951.
15. Ochoa, S. and Stern, J. R. Carbohydrate metabolism. Ann. Rev. of Biochem. **21**: 547–602. 1952.
16. Roe, J. A colorimetric method for the determination of fructose in blood and urine. Jour. Biol. Chem. **107**: 15–22. 1934.
17. Sable, H. Z. Pentose metabolism in extracts of yeast and mammalian tissues. Biochim. Biophys. Acta **8**: 687–697. 1952.
18. Seegmiller, G. E. and Horecker, B. L. The synthesis of glucose-6-phosphate and 6-phosphogluconate. Jour. Biol. Chem. **192**: 175–180. 1951.
19. Warburg, O. and Christian, W. Isolieung und Kristallisation des Garungsferments Enolase. Biochem. Z. **310**: 384–421. 1942.

BRIEF PAPERS

FRENCHING OF RAGWEED (*AMBROSIA ARTEMISIIFOLIA* L.)[1]

LINUS H. JONES

DEPARTMENT OF BOTANY, UNIVERSITY OF MASSACHUSETTS AGRICULTURAL EXPERIMENT STATION, AMHERST, MASSACHUSETTS

Received August 9, 1951

Typical frenching of tobacco (*Nicotiana tabacum* L.), as described by WOLF (**8**), is recognized as producing short internodes, loss of apical dominance, and the development of a rosette of many leaves (polyphylly), giving a witches'-broom effect. The first symptoms appear as a pinhead chlorosis, followed by very chlorotic, newly developing leaves which narrowly elongate with wavy margins. It is possible that the tobacco plant (*Nicotiana tabacum* L.) is the best indicator plant to determine the presence of the frenching factor in the soil. This peculiar development of the leaves of tobacco has never been found to be associated with a pathogenic bacterium, a fungus, or a virus. It is generally recognized as a nonparasitic disease and therefore considered physiological. STEINBERG (**5, 6**) has worked with many tobacco soil organisms which are not considered pathogenic but which might produce a diffusate sufficiently toxic to produce the symptoms of frenching. One of these, *Bacillus cereus* Frankland and Frankland, was found to be capable of producing the frenching symptoms. STEINBERG (**7**) has found this organism to be present in greater numbers in the rhizosphere of tobacco in soils that contained frenched tobacco than in the soils with normal plants.

The origin of this disease is some soil factor. The frenching factor is not in all soils but has been found, in particular, in soils which are known to remain moist during a prolonged drought period, even though tobacco has never been grown in such soils.

Continuing the work previously reported (**2, 3**), it has been demonstrated that the frenching factor, if present, can be made to show its presence by growing tobacco plants at a soil temperature of 35° C. Usually within a period of 5 to 15 days frenching symptoms appear in the newly developing leaves, becoming more distinct and severe with succeeding leaves. When the soil temperature is 21° C, or less, the presence of the frenching factor does not become evident.

During the investigation of the relationship of soil temperature to the frenching factor that affects tobacco, many volunteer weed plants of various genera appeared in the soils employed in the study. Ragweed (*Ambrosia artemisiifolia* L.), as a weed, frequently showed chlorotic symptoms on leaves that were so narrow they appeared like starched string. These symp-

[1] Published as contribution no. 813 of the Massachusetts Agricultural Experiment Station.

toms developed only from soils that produced frenching of tobacco. The same soil at the lower temperature of 21° C produced neither tobacco frenching nor any abnormal specimens of ragweed comparable to those found at the higher soil temperature of 35° C.

Ragweed was tested in the laboratory greenhouse under different seasonal conditions of length of day and in different frenching soils to demonstrate that it can be induced to french by the soil temperature technique. The early symptoms of frenching, a chlorotic leaf with very narrow leaflets, suggested aster yellows caused by a virus common to ragweed. Aster yellows was ruled out by an expert on virus diseases, particularly aster yellows.

Young or seedling ragweed plants were obtained by skimming the soil where ragweed was prolific and sowing these skimmings on a flat of autoclaved soil. Autoclaving of soil destroys the frenching factor (**4**). In the winter, skimmings were obtained along the edge of a cinder path where ragweed was common during the summer. Uniform young plants were pricked out and allowed to establish themselves in the testing soil before being subjected to the desired soil temperature. The general plan for each experiment consisted of eight two-gallon containers which could be placed in a water tank for the desired temperature. In each container were planted three tobacco and three ragweed plants. The tobacco plants could be easily removed by cutting at the soil line, after frenching, to provide space and light for the slower growing ragweed. Four containers were used at each temperature, but one of these always contained a nonfrenching soil to prove and demonstrate that soil temperature alone is not the direct cause of frenching.

In one experiment the tobacco alongside ragweed frenched in 12 days, but the first signs of chlorosis on ragweed were not apparent until the nineteenth day. The most distinct symptoms were present about the end of the fifth week. At this time the bipinnatifid leaf was greenish yellow in color with the segments inflexed on the ventral surface. A high level of soil fertility was maintained throughout this as well as the other experiments. The plants were frequently tested with diphenylamine (**1**) for nitrates, and in no cases were nitrates absent. Thus the slower growth and chlorotic condition of leaves when compared with the checks at a soil temperature of 21° C could not be ascribed to a lack of nitrogen in the soil or in the plant.

In another experiment in which frenching was slow to develop in tobacco, 20 to 35 days, it took 25 to 34 days for all the nine ragweed plants to show frenching. When the nonfrenched ragweed plants at the soil temperature of 21° C were subjected to the higher soil temperature of 35° C, one plant frenched in five days; seven, within a period of 12 days; and the last two, 16 and 22 days, respectively. On the other hand, those plants that had frenched at the higher soil temperature of 35° C gradually outgrew the tendency to french when the soil temperature was lowered to 21° C. These plants developed axil and terminal shoots, the lower leaves of which were frenched; but as succeeding leaves developed, they were constantly less frenched and finally developed normal leaves. This lag follows the same pattern as that noted with tobacco (**2**).

The nonfrenching soil at the higher temperature of 35° C gave no evidence of producing frenching of tobacco or chlorosis of ragweed; neither did its corresponding check at 21° C when it was subjected to the higher temperature. Not only does this confirm the previous experiences with tobacco but it also indicates that ragweed in order to show signs of frenching must be grown in a soil in which the frenching factor is present and subjected to a relatively high soil temperature. Soil temperature by itself is not the frenching factor. Figure 1 illustrates normal, severely frenched, and moderately frenched plants. The middle specimen is so frenched the leaves are of stringlike form.

Ragweed is not so satisfactory as tobacco to demonstrate the presence of the frenching factor in the soil. Some ragweed plants do not maintain the frenching symptoms, whereas other ragweed plants add to the symp-

FIG. 1. Frenching in ragweed (*Ambrosia artemisiifolia* L.). Left to right: normal, severe (stringlike leaves), and moderate.

toms by developing side shoots with short stems and accentuated frenching symptoms. This side shoot development follows the loss of apical dominance of a shoot similar to axillary bud development in tobacco. In both ragweed and tobacco this axillary bud development is more extended than if the plants had been mechanically topped, for it may extend to the lowest axils on the plants.

Sorrel (*Oxalis stricta* L.) was another weed that was susceptible to the frenching factor at a relatively high soil temperature, 35° C. No symptoms of frenching appeared on the volunteer weed plants, Galinsoga (*Galinsoga parviflora* L.), smartweed (*Polygonum pensylvanicum* L.), purslane (*Portulaca oleracea* L.), and several genera of the grass family. In addition to tobacco, frenching symptoms could be obtained in tomato (*Lycopersicum esculentum* Mill. var. John Baer) and squash (*Cucurbita maxima* Duchesne var. Blue Hubbard), but not in pepper (*Capsicum frutescens* L. var. California Wonder). All these plants were grown and observed alongside to-

bacco plants that had frenched and were, therefore, subjected to the same factor in the soil that caused tobacco frenching.

Tobacco and tomato are both solanaceous plants and both will french. Pepper is also a solanaceous plant but it has shown no evidence of frenching. The other plants that frenched (sorrel, ragweed and squash) are widely separated from tobacco in any taxonomic list and also widely separated from each other. It thus becomes impossible to determine from a taxonomic guide what plants are susceptible to the frenching factor.

The frenching symptoms of tobacco were once thought to be of virus origin, and it is possible that some of the plants listed as susceptible to frenching were affected by a virus. One plant of an old list of suscepts, petunia (*Petunia hybrida* Hort.) was tested by the soil temperature technique for inducing frenching. No general symptoms appeared that might have been called frenching, but plants at both the high and low soil temperatures were chlorotic and resembled virus-infected plants. The suggested technique of using a relatively high soil temperature with a soil known to contain the frenching factor is a possible procedure for separating and studying the symptoms of true frenching without confusing them with other diseases that produce a chlorotic condition with malformed leaves.

The author is indebted to Dr. L. O. Kunkel of the Rockefeller Institute for Medical Research for examining specimens of ragweed and confirming the diagnosis that the chlorotic symptoms were caused by a frenching factor and not by the virus causing aster yellows.

LITERATURE CITED

1. Jones, L. H. Diagnosis of plant troubles with diphenylamine. Plant Physiol. **11**: 207–209. 1936.
2. Jones, L. H. Soil temperature as a factor in the frenching of tobacco (*Nicotiana tabacum* L.). Plant Physiol. **23**: 560–575. 1948.
3. Jones, L. H. and Tio, M. A. Unavailability of iron as a cause of frenching of tobacco (*Nicotiana tabacum* L.). Plant Physiol. **23**: 576–594. 1948.
4. Shear, G. M. Field and laboratory studies of frenching of tobacco. Virginia Agr. Exp. Sta. Tech. Bull. 49. 1933.
5. Steinberg, R. A. Symptoms of amino acid action on tobacco seedlings in aseptic culture. Jour. Agr. Res. **78**: 733–741. 1949.
6. Steinberg, R. A. The relation of certain soil bacteria to frenching symptoms of tobacco. Bull. Torr. Bot. Club **77**: 38–44. 1950.
7. Steinberg, R. A. Occurrence of *Bacillus cereus* in Maryland soils with frenched tobacco. Plant Physiol. **26**: 807–811. 1951.
8. Wolf, F. A. Tobacco Diseases and Decays. Duke University Press, Durham, North Carolina. 1935.

EFFECT OF STABLE BORON ISOTOPES ON GROWTH OF TOBACCO IN SOLUTION CULTURES

J. E. McMURTREY, JR. AND H. B. ENGLE

BUREAU OF PLANT INDUSTRY, BELTSVILLE, MARYLAND

Received December 20, 1951

It has been stated and is generally accepted that the isotopes whether stable or radioactive behave relative to biological functions as do the commonly occurring forms. It appears to have been assumed that they will function in all phases in the physiology of a green plant in the same way. However, lethal effects (**7**) and other effects (**1**) on the plants have been reported but appear to be associated with the level of radioactive material to which the plant is exposed. There were some reductions in growth even when the concentration was below what was considered the critical level. These findings have been supported by recent work (**2**).

The extensive studies (**3**) now under way with radioactive isotopes of the elements essential to plant growth including carbon, calcium, nitrogen and phosphorus, would appear to be intimately related to the assumption that they function biologically as do the commonly occurring forms.

The micronutrient elements have not apparently received any considerable attention. One paper by STOUT and MEAGHER (**8**) deals with radioactive molybdenum effects in plant growth. No publication dealing with stable or radioactive isotopes of boron effects on plant growth has been noted.

The stable isotope of boron, B^{10}, recently made available by the Atomic Energy Commission appeared to offer an interesting approach to study any differences in growth from stable boron isotopes. Comparisons using boric acid commonly available as the reagent grade and boric acid prepared from the complex supplied by the commission in which 96% of the boron occurred as B^{10} were carried out in solution cultures using tobacco as the test plant.

The cultures were located on three adjacent tables with the B^{11} groups on one table and the B^{10} groups on another with the groups with no boron on a third table placed between the two. The tables were 13.5 feet long and one foot seven inches wide and were located one foot 10.5 inches apart. The plants were thus grown in an area 8.5 feet by 13.5 feet so that if there were a position effect on growth it should not be of a serious nature.

The procedures used in culturing the plants were essentially those described previously (**5, 6**). The solutions used in these studies were of the same composition as those previously used (**6**). They contained the following volume molecular concentrations: $Ca(NO_3)_2 \cdot 4H_2O$, 0.00617; KNO_3, 0.00108; $Mg(NO_3)_2 \cdot 6H_2O$, 0.00062; KH_2PO_4, 0.00211; and NH_4Cl, 0.00140. The parts per million of the various elements supplied were: Nitrogen, 225; phosphorus, 66; potassium, 125; calcium, 247; magnesium, 30; sulphur, 20; and chlorine, 50. The following elements were supplied in parts per million

in the amounts indicated as sulphates. Copper, 0.125; manganese, 1.0; and zinc, 0.5. Iron as the sulphate was added to supply 1.0 p.p.m. of this element on April 8, the starting date of the experiment, and thereafter on April 11, 12, 13, 14, 15 and 22.

The Connecticut Broadleaf variety of tobacco was used in these studies. The seeds were sown in a flat of steam-sterilized greenhouse potting soil mixture, and after approximately one month they were transplanted to two-inch pots containing the same soil sterilized in the same manner. After the plants were allowed to develop in these pots for about two weeks the roots were washed free of soil, and the plants were transferred to the starter solution without covers as described (**4**) for about two weeks. When the largest leaves were about three and one half by seven inches the plants were placed in the experimental solutions.

The variable under investigation consisted of a comparison of boron isotopes as a source of the element. In one group the boron was derived from reagent grade of boric acid, which is made up of the usual occurring proportions of the isotopes of boron, 18.4% B^{10} and 81.6% B^{11}, which is referred to in the following discussions as B^{11}. Another lot was made up from that containing 96% B^{10} and 4% B^{11} and referred to in the following discussion as B^{10}. All calculations were made on the basis of above analyses to supply a given amount of boron atoms, but are referred to below as the equivalent concentrations that would have been supplied by natural boron.

There were five groups of treatments. One group of 12 plants received no boron. One group of six plants received 0.1 p.p.m. B^{11}. One group of six plants received 0.5 p.p.m. B^{11}. One group of six plants received 0.1 p.p.m. B^{10}. One group of six plants received 0.5 p.p.m. B^{10}.

The plants were placed in the experimental solutions April 8, 1949, and on April 14 the first sign of boron deficiency was evident on one plant of the 12 growing in the solution to which no boron was added. The base of the younger leaves making up the terminal bud showed a faint light green color. The next leaf below was brittle and broke when handled. At this date there was no apparent evidence of abnormality in any other plants.

On the tenth day after the start of the experiment all twelve plants that were growing in the solution to which no boron was added showed characteristic boron deficiency symptoms culminating in breakdown of the terminal bud. Also, one plant receiving 0.1 p.p.m. B^{11} showed the first indication of boron deficiency 10 days after transfer to the experimental treatment. After 18 days, a second plant receiving 0.1 p.p.m. B^{11} was showing deficiency symptoms, while none of those receiving 0.1 p.p.m. B^{10} showed any symptoms, and appeared to be growing more uniformly than those receiving B^{11}. There was no abnormality in any plants receiving 0.5 p.p.m. boron in either form. After 21 days, five plants out of six with 0.1 p.p.m. B^{11} were showing boron deficiency, while two out of six with 0.1 p.p.m. B^{10} were showing deficiency. After 25 days, all plants with 0.1 p.p.m. B^{11} showed deficiency, and four out of six with 0.1 p.p.m. B^{10} showed deficiency. After 28 days,

the remaining two plants in the 0.1 p.p.m. B^{10} solution showed the characteristic breakdown symptoms associated with boron deficiency. All plants receiving 0.5 p.p.m. boron in either form showed no abnormality and were starting to flower.

All cultures received 100 cc. of stock nutrient solution three weeks after the start of the experiment. This was added to the solutions in which the plants were growing and no aditional micro-elements were added. On the twenty-eighth day (May 6) all plants were harvested and hung in the greenhouse to air-dry.

The results from this test are shown in table I. The air-dry weight of leaf produced from the two rates and sources of boron are in close agreement. There was a striking increase in yield of leaf when boron was used either as B^{10} or B^{11} over that obtained when no boron was used. The most striking effect is manifested by the stalk weights when the no-boron treatment is contrasted with the two rates and sources. There was also a decided

TABLE I

AVERAGE AIR-DRY WEIGHT PER PLANT FOR TOBACCO GROWN IN NUTRIENT SOLUTIONS WITH TWO FORMS AND RATES OF BORON.

Boron		Air-dry weight				Standard error of total
Form	Rate	Leaves	Stalk	Roots	Total	
	p.p.m.	*gm.*	*gm.*	*gm.*	*gm.*	
None	None	6.18	1.07	1.49	8.74	± 2.69
B^{11}	0.1	13.66	4.18	3.69	21.53	± 2.77
B^{10}	0.1	14.77	6.68	3.88	25.33	± 1.38
B^{11}	0.5	14.33	9.84	4.43	28.60	± 2.36
B^{10}	0.5	14.50	10.13	4.49	29.12	± 1.48

increase in the air-dry weight of stalks with the higher compared to the lower rate of boron. There appears to be a slight advantage for the B^{10} treatment of a given concentration in yield of stalk although the statistical significance of this difference is questionable. The yield of roots was greatly increased by both forms of boron at the two rates as contrasted with the no-boron treatment. The higher rate of boron has produced a higher air-dry weight of roots. The total air-dry weight shows much the same trends as that of the stalks. There is a decided increase in total yield for both forms and rates of boron. The higher rate of boron has resulted in a significantly higher yield than the lower rate. As previously pointed out, this difference is largely the result of a higher yield of stalk.

In every instance, the total yields are higher from the B^{10} than from the B^{11} but the difference, based on weights of individual plants, was not found to be statistically significant at the higher concentration. The difference appears to be significant at the lower concentration. During the growth of the plants there was no observable difference in growth effects from the two isotopes of boron with the exception of that previously noted, namely, the

earlier occurrence of deficiency symptoms where the low rate (0.1 p.p.m.) B^{11} was used. This difference was not due to extra molecules of boron from the B^{10} source since the two forms were added to supply an equal number of molecules of each form. Actually there was less weight added in the B^{10} form than in the B^{11} form.

It would appear from this test that B^{10} produces essentially the same growth responses as B^{11} in tobacco plants when used to supply the element boron in solution cultures.

The stable isotope of boron was secured from the Isotopes Division, Atomic Energy Commission, as the BF_3CaF_2 complex containing 6.9% of elemental B of which 96% was B^{10}. Mr. Frank O. Lundstrom of the Bureau's Division of Fertilizer and Agricultural Lime kindly converted this compound to boric acid according to the method of Dr. Walter B. Keighton.

LITERATURE CITED

1. Arnason, T. J., Cumming, Elaine, and Spinks, J. W. T. Chromosome breakage induced by absorbed radioactive phosphorus P^{32}. Canadian Jour. Res. **26C**: 109–114. 1948.
2. Blume, J. M., Hagen, C. E., and Mackie, Ruth M. Radiation injury to plants grown in nutrient solutions containing P^{32}. Soil Science **70**: 415–426. 1950.
3. Burris, R. H. Isotopes as tracers in plants. Bot. Review **16**: 150–180. 1950.
4. Engle, H. B. and McMurtrey, J. E., Jr. Effect of algae in relation to aeration, light and sources of phosphorus on growth of tobacco in solution cultures. Jour. Agr. Res. **60**: 487–502. 1940.
5. McMurtrey, J. E., Jr. The effect of boron deficiency on the growth of tobacco plants in aerated and unaerated solutions. Jour. Agr. Res. **38**: 371–380. 1929.
6. McMurtrey, J. E., Jr. Distinctive effects of the deficiency of certain essential elements on the growth of tobacco plants in solution cultures. U. S. Dept. Agr. Tech. Bull. 340. 1933.
7. Spinks, J. W. T., Cumming, Elaine, Irwin, L. B., and Arnason, T. J. Lethal effect of absorbed radio-isotopes on plants. Canadian Jour. Res. **26C**: 249–262. 1948.
8. Stout, P. R. and Meagher, W. R. Studies of molybdenum nutrition of plants with radioactive molybdenum. Science **108**: 471–473. 1948.

LOW TEMPERATURE INDUCTION OF FLOWERING IN A *NICOTIANA RUSTICA* × *N. TABACUM* HYBRID

ROBERT A. STEINBERG

BUREAU OF PLANT INDUSTRY, BELTSVILLE, MARYLAND

Received May 23, 1952

One of the Nicotiana varieties propagated and studied in this division for many years has become known as mammoth or non-flowering rustica. It originated as a cross between *Nicotiana rustica* Link and a presumed short-day mammoth variety of *N. tabacum* Link. By repeated back-crossing to *N. rustica* and selecting for mammoth types of growth, a variety was obtained that formed a large number of leaves and grew to be very tall (indeterminate growth). Unlike the true mammoth type of tobacco (**1**), however, only rarely would a plant of this rustica variety flower even during the short days of winter in the greenhouse. SMITH (**2**) has called attention to these facts and has suggested that a possible explanation may consist in a modified reaction of the short-day mammoth gene of tobacco in the *N. rustica* genetic matrix. Propagation of this mammoth-rustica variety has usually been by means of cuttings.

Many unsuccessful expedients have been tried by the writer in attempts to induce flowering in this variety. These included clone selection, day lengths from 2 to 12 hours, drought, darkness, defoliation, root exposure, use of very limited quantities of soil, and triiodobenzoic acid. Flowering has not been observed in field studies, but this may be because it is usually necessary to decapitate and harvest the plants before advent of frost.

This variety was of interest for two reasons. It was desirable to work out a method to obtain sufficient seed for use in field studies on nicotine production and as material in breeding studies for obtaining varieties high in nicotine.

The mammoth-rustica plants were obtained either from seedlings or cuttings. Cuttings were made from suckers produced by decapitating plants about 18 inches tall. These were transplanted to one-gallon glazed crocks. A good quality of potting soil was used and was usually supplemented by addition of 5 gm. of 4–8–12 fertilizer to each crock every 7 to 10 days in the later stages of growth. A temperature of 75° F was maintained in the warm section both day and night. Temperatures in the cold section of the greenhouse were 65 to 75° F during the day, and 50 to 60° F at night.

The first indication of the major cause for sterility of this strain came during the winter of 1946. Because of a coal scarcity it was necessary to lower temperatures in the greenhouse, previously maintained at 75° F, for a week or more. Night temperatures were, therefore, dropped to about 45° F and day temperature to 60 to 65° F. Of a batch of 16 plants then growing in the greenhouse in galvanized-iron buckets, four flowered and produced seed.

TABLE I

FLOWERING BEHAVIOR IN 1949 OF SUCCESSIVE LOTS OF MAMMOTH-RUSTICA PLANTS ROOTED IN A WARM SECTION AND LATER TRANSFERRED TO A COLD SECTION.

Placed in cool section	Total plants	Number flowering	Flowering
			%
August 15	47	46	97.9
October 20	19	2	10.5
November 1	26	5	19.2
November 7	33	32	97.0
November 15	18	2	11.1
December 28	20	10	50.0

In the winter of 1949 lots of plants were started at various times by cuttings from a mixture of clones transplanted to four-inch pots and shifted to the cool section at irregular intervals. The data on flowering are shown in table I. Flowering of the different lots ranged from 10.5 to 97.9%, and much seed was obtained. The irregularity in quantitative response was assumed to be due to differences in time between rooting of the cuttings in the warm section and their transfer to the cold section. That this was not the entire explanation and that genetic variability was also an important factor was demonstrated in the next trial.

A more systematic test was carried out the following winter. Both seed and cuttings were started in the cold section and later transplanted to one-gallon glazed crocks. There were nine plants per lot. The seed consisted of a miscellaneous mixed lot collected over a period of many years from occasional plants that had fruited. The cuttings were from a single clone, selected because it seemed incapable of flowering.

Flowering of plants propagated by either seed or cuttings was quite profuse (table II). Flowering was complete with plants started as cuttings

TABLE II

FLOWERING BEHAVIOR IN 1950 OF SUCCESSIVE LOTS OF NINE PLANTS OF MAMMOTH-RUSTICA PLANTS GROWN AT COLD TEMPERATURES.

Date of sowing and setting cuttings	Flowering of plants from single clone				Flowering of plants from miscellaneous bulked seed			
	Plants	Days	Range	Height	Plants	Days	Range	Height
		aver.	*days*	*ins.*		*aver.*	*days*	*ins.*
August 1	9	164	6	87	9	88	91	38
September 1	9	139	15	79	8	113	64	54
October 1	9	154	38	80	9	150	56	76
November 1	9	147	18	73	6	147	53	66
December 1	9	139	14	71	4	137	94	49
January 1	9	117	16	53	8	126	90	47
February 1	6	120	45	45	5	157	46	70
March 1	1	97		38	6	122	49	43
April 1	1	112		59	8	117	89	56

over a period of six months and only diminished with the February, March, and April lots when greenhouse temperatures could not be controlled as well as in the previous months. Flowering of the plants grown from seed was more irregular than that from cuttings, and did not decrease to a similar extent at any time. Blossoming occurred from October to August, inclusive, in the various lots of plants. Plants in the last lot that was started on April 1 were subjected to day lengths of over 12 hours at all times, but one plant from the clone and eight of those from seed flowered into July and August. Another feature of interest in this connection is the much greater variability in flowering of individual plants, of the lots grown from seed than from cuttings. Moreover, while plants from the clone formed small pods no seed was formed, whereas many of the plants started from seed set seed readily.

A final comparison between the effects of warm and cold temperature on blossoming was made the following year. All plants were from the same clone, and the cuttings were started at the same time (September 1). Of 36 plants grown in the warm section, none had blossomed on March 8. They averaged 76 inches in height at this time, though many of the stems had snapped. Of the 36 plants grown in the cold section, all had blossomed by this date. The average for time of blossoming was 148 days; range in blossoming of individual plants, 54 days; and average height at blossoming was 89 inches.

The plants at one end of two benches in the cold section had been exposed to spillage of light from the next section, where high wattage tungsten lamps were being used to extend the day length to about 18 hours (sunset to midnight). No difference in time of blossoming of the plants on the bench farthest from the lights could be detected. A few of the plants nearest the lamps on the closer bench flowered somewhat later. Of these six late-flowering plants, two were abnormal and had not been expected to flower. These six late-flowering plants blossomed on the average in 177 days, over a period of 33 days. The other thirty plants of the lot had bloomed between January 14 to 30, a range of 16 days. The average for flowering of these 30 plants was 143 days, as compared to 139 days in the previous experiment.

It is evident from these data that the mammoth-rustica plant is not of true mammoth type since it is not characterized by a specific requirement of short days for flowering. Plants have been observed to flower even when grown continuously under day lengths ranging from 12.5 hours to 15.5 hours, exclusive of the two twilight periods. Its occasional responses to winter conditions in the greenhouse have probably been caused by unnoted drops in temperature. An additional factor that may have contributed to the irregular and infrequently observed flowering may be the inherent lack of genetic uniformity of this variety. Plants from seed did not respond uniformly to variations in temperature as respects flowering. Many strains could probably be selected from this variety on the basis of flowering and seeding

responses to temperature. This viewpoint is borne out by differences in inflorescence of the plants. Early flowering plants usually formed terminal panicles. Late flowering plants bore blossoms on very short axillary branches beginning with or near the terminal bud (racemes). The inflorescences of plants from the clone selected for these experiments were also racemes, and never panicles.

Plants of Maryland Mammoth tobacco were grown at times in both cold and warm sections during these experiments. They behaved in all respects as described by GARNER and ALLARD (**1**). All flowered and seeded freely during the short days of winter even though the minimum temperature in the warm section was 75° F, and that in the cold section was 50° F. The lower temperature delayed but did not diminish blossoming. With the increasing day lengths of spring, the seedlings could no longer flower, but became indeterminate in growth habit. The possibility exists, therefore, that there may be two types of mammoth tobacco both of which are characterized by gigantism. One, as in Maryland Mammoth, possesses the additional property of giving a short-day-length flowering response; another, a low-temperature flowering response. Indeterminate growth actually would be due to non-flowering caused by unfavorable day lengths and unfavorable temperatures, respectively.

If the assumption of the presence of a true short-day mammoth gene from tobacco (**2**) in this rustica variety is correct, it must be concluded that it no longer behaves as a day-length flowering preceptor, but as a temperature flowering preceptor. However, a check for the presence of the short-day mammoth gene in this variety by its transfer back to tobacco (preferably Maryland Medium Broadleaf) would seem desirable.

Mammoth-rustica, it seems, exemplifies a new type of flowering response in Nicotiana species. A low temperature requirement for blossoming has hitherto been associated primarily with certain long-day biennial forms. Day-neutral Nicotiana plants, it now appears, may also possess a low temperature requirement for flowering.

LITERATURE CITED

1. GARNER, W. W. and ALLARD, H. A. Effect of relative length of day and night and other factors of the environment on growth and reproduction in plants. Jour. Agr. Res. **18**: 553–606. 1920.
2. SMITH, H. H. Differential photoperiod response resulting from interspecific gene transfer. Amer. Jour. Bot. **36**: 828–829. 1949.

THE EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON PHOSPHORYLASE, PHOSPHATASE, AMYLASE, CATALASE, AND PEROXIDASE ACTIVITY IN WHEAT

D. J. WORT AND L. M. COWIE

DEPARTMENT OF BOTANY, UNIVERSITY OF BRITISH COLUMBIA, VANCOUVER, BRITISH COLUMBIA

Received February 11, 1952

The changes in carbohydrate, nitrogen, protein, and amino acid content and in respiratory rates of plants and plant parts treated with 2,4-dichlorophenoxyacetic acid (2,4-D) indicate effects of the chemical at enzyme levels. Some of these effects have been investigated. NEELY *et al.* (**6, 7**) found lowered activity of phosphorylase and beta-amylase but increased activity of pectin methoxylase in treated kidney bean plants. Increases in peroxidase activity in kidney bean leaf protuberances caused by 2,4-D application were obtained by FELBER (**2**). Growth of corn and cowpea seedlings in a solution of 1.5 p.p.m. of 2,4-D resulted in an increased activity of phosphatase in the roots, compared with controls, according to OLSEN (**8**). HAGEN *et al.* (**5**) state that 2,4-D produces no inhibition of polyphenoloxidase, alpha-hydroxy acid oxidase, or catalase in castor bean, using the tissue homogenate technique. The same authors report that the activity of lipase in this plant is depressed by 2,4-D at very low concentrations. GOLDACRE (**3**) found that concentrations of up to 2000 mg. of 2,4-D per liter have a stimulatory effect on the activity of indoleacetic acid oxidase from pea stems.

In previous work at this laboratory (**11, 12**), the changes in chemical composition of plants treated with lethal and non-lethal concentrations of 2,4-D were studied at intervals over a period of time following treatment. Since similar fluctuations in activity of appropriate enzymes might occur, an investigation was initiated to determine the apparent phosphorylase, phosphatase, and beta-amylase activity at intervals following the application of non-lethal concentrations of 2,4-D to wheat. A study of catalase and peroxidase activity was also made.

Marquis spring wheat was planted in the greenhouse on April 21 and on June 15, 1951. Because of the similarity of results, the June experiment is described below. Twenty-four days after sowing, the plants, now 10 inches high, were sprayed with an aqueous solution of 5 or 500 p.p.m. diethanol amine of 2,4-D. Unsprayed plants served as controls. Nine hours, 1, 2, 4, and 8 days after spraying, 180 plants from each treatment and from the control group were cut at the soil surface, the stems and leaves were chopped into small pieces and mixed thoroughly. Aliquots of 40 gm. were blended with 100 ml. of ice water for two minutes and filtered through broadcloth. The filtrate was used immediately to determine the apparent activity of the

five enzymes. The spraying and all harvests, except that made nine hours after treatment, were made at 10 A.M.

Phosphorylase activity was measured by the method of SUMNER *et al.* (**10**), beta-amylase by SUMNER and SOMER'S modification (**9**) of the procedures of Willstatter and coworkers, phosphatase by GOTTSCHALK'S micromethod (**4**), catalase by SUMNER and SOMER'S (**9**) modification of the procedure of von Euler and Josephson, using three- and six-minute intervals, and peroxidase by the method of DAVIS (**1**). Effects of 2,4-D added to the filtrate of untreated plants in the same concentration as the concentration computed for the filtrates of treated plants, assuming that all the 2,4-D sprayed on the plants was absorbed, were determined and found to lack significance.

The values for the activities of the various enzymes and the T/C values (values obtained for apparent activities in the filtrates of the treated plants, divided by the values in the control plants) are given in table I. The values for phosphorylase, expressed as micrograms of phosphorus formed per ml. of extract, show that sprays of 5 and 500 p.p.m. produced an immediate increase in phosphorylase activity. The activity diminished as all the plants aged, but the levels in the treated plants were higher at all times. The dose of 500 p.p.m. produced the greater increase in activity. The highest activity value in both control and treated plants was measured nine hours after treatment. This observation suggests that phosphorylase activity may be linked with time of day, since the collection was the only one made in the evening. All other harvests were made at 10 A.M. The greatest T/C values, 2.34 for 5 p.p.m. and 2.14 for 500 p.p.m. 2,4-D, were obtained one day after spraying.

Phosphatase activity was measured as micrograms of phenol formed per milliliter of plant extract. There was considerable activity of this enzyme in both control and treated plants. The application of both 5 and 500 p.p.m. of 2,4-D depressed the activity slightly, the effect being more pronounced with the larger concentration. The T/C values fell to minima of 0.8 one day after spraying and remained below 1.0 for the duration of the experiment.

The monomolecular reaction constant for beta-amylase was obtained from the equation:

$$K = 1/t \log \frac{0.1875}{0.1875 - x}$$

The time in minutes is represented by t, and x is the corrected titration value $\times$ 17.15 (**9**). The activity values for beta-amylase remained relatively constant in controls during the experiment, but up to the fourth day after spraying considerable increases over control values resulted from treatment with 2,4-D. Between the fourth and eighth days there were decreases in activity in the treated plants which resulted in final T/C values of 0.66 and 0.53 for sprays of 5 and 500 p.p.m. respectively. At comparable times after spraying the results were similar to those reported for kidney beans (**7**).

TABLE I

PHOSPHORYLASE, PHOSPHATASE, BETA-AMYLASE, CATALASE, AND PEROXIDASE ACTIVITY IN MARQUIS WHEAT PLANTS SPRAYED WITH 2,4-D.

Time after spraying	Enzyme activity			T/C values	
	Control plants	5 p.p.m. 2,4-D	500 p.p.m. 2,4-D	5 p.p.m. 2,4-D	500 p.p.m. 2,4-D
			Phosphorylase		
9 hours	8.16	9.73	10.12	1.19	1.24
1 day	3.27	7.65	7.02	2.34	2.14
2 days	5.75	7.28	9.29	1.26	1.61
4 days	5.13	6.16	8.12	1.20	1.58
8 days	3.36	3.64	4.91	1.08	1.40
			Phosphatase		
9 hours	288.6	286.0	273.0	0.99	0.95
1 day	327.6	287.3	273.0	0.81	0.80
2 days	282.1	263.9	236.6	0.96	0.84
4 days	278.2	254.8	239.2	0.92	0.86
8 days	240.5	215.8	198.9	0.90	0.83
			Beta-amylase		
9 hours	1.798×10^{-3}	2.250×10^{-3}	2.437×10^{-3}	1.24	1.34
1 day	1.166×10^{-3}	1.635×10^{-3}	2.282×10^{-3}	1.40	1.95
2 days	2.141×10^{-3}	2.475×10^{-3}	3.068×10^{-3}	1.11	1.43
4 days	1.836×10^{-3}	2.039×10^{-3}	2.671×10^{-3}	1.11	1.40
8 days	1.937×10^{-3}	1.276×10^{-3}	1.032×10^{-3}	0.66	0.53
			Catalase		
9 hours	0.0443	0.0465	0.0478	1.05	1.08
1 day	0.0296	0.0488	0.0477	1.65	1.61
2 days	0.0256	0.0422	0.0271	1.65	1.06
4 days	0.0281	0.0294	0.0253	1.03	0.90
8 days	0.0223	0.0252	0.0267	1.13	1.19
			Peroxidase		
9 hours	1.73×10^{-3}	1.62×10^{-3}	1.50×10^{-3}	0.93	0.87
1 day	1.10×10^{-3}	1.37×10^{-3}	1.42×10^{-3}	1.24	1.29
2 days	1.63×10^{-3}	1.66×10^{-3}	1.63×10^{-3}	1.02	1.00
4 days	1.09×10^{-3}	1.19×10^{-3}	1.25×10^{-3}	1.09	1.15
8 days	0.88×10^{-3}	0.79×10^{-3}	0.76×10^{-3}	0.90	0.87

Catalase activity is given in the table as the monomolecular value extrapolated to zero time. The activity of the enzyme was increased as a result of treatment with 2,4-D. The greatest relative increase occurred one and two days after the application of the chemical, rising to over 1.6 with both sprays. The values of T/C fell to control levels by the fourth day but rose above 1.0 by the final day of the experiment.

To express peroxidase values the reciprocal of the time for the color change to occur in the blank was subtracted from the reciprocal of the time required in the active solution (**1**). The activities in the treated plants paralleled those in the controls but remained greater than those of controls up to the fourth day after 2,4-D was applied. The greatest relative activi-

ties occurred one day after the application of the chemical, being 1.24 or over, but the T/C values fell below 1.0 by the eighth day. The effects of the sprays of 5 and 500 p.p.m. were similar.

An examination of the curves of values of T/C for phosphorylase, beta-amylase, peroxidase and catalase activity in wheat given non-lethal concentrations of 2,4-D reveals trends very similar to those for the total sugar, starch-dextrin, available carbohydrates and total N content of stems of buckwheat sprayed with lethal doses (**11**) and those for total sugar and starch-dextrin in buckwheat which received non-lethal doses of the chemical (**12**). These show an increase over control levels soon after spraying, followed by a return to normal or below normal several days after treatment. Whether the influence of 2,4-D on the activity of enzymes is brought about by its direct action on the prosthetic group or the protein portion of the enzyme, or indirectly on the availability of amino acids and vitamins for the elaboration of the enzymes by the plant has yet to be determined.

In the experiments reported above, spraying Marquis wheat plants with 5 or 500 p.p.m. of 2,4-D resulted in increases in the activity of phosphorylase, beta-amylase, catalase, and peroxidase in the aerial portions of the plant. The activity of phosphatase was decreased by the treatment. The deviations from the control levels reached maxima by the first or second day after treatment.

This study was supported in part by a grant from the Research Fund of the University of British Columbia.

LITERATURE CITED

1. Davis, W. B. Quantitative field test for estimation of peroxidase. Ind. Eng. Chem., Anal. Ed. **14**: 952–953. 1942.
2. Felber, I. M. The formation of protuberances of bean leaves in response to 2,4-D treatments. Amer. Jour. Bot. **35**: 555–558. 1948.
3. Goldacre, P. L. On the mechanism of action of 2,4-dichlorophenoxyacetic acid. Australian Jour. Sci. Res. B **2**: 154–156. 1949.
4. Gottschalk, R. G. Microdetermination of acid phosphatase. Biochim. Biophys. Acta **2**: 582–589. 1948.
5. Hagen, O. E., Clagett, C. O., and Helgeson, E. A. 2,4-D inhibition of castor bean lipase. Science **110**: 116–117. 1949.
6. Neely, W. B., Ball, C. D., Hamner, C. L., and Sell, H. M. Effect of 2,4-dichlorophenoxyacetic acid on the invertase, phosphorylase, and pectin methoxylase activity in the stem and leaves of red kidney bean plants. Plant Physiol. **25**: 525–528. 1950.
7. Neely, W. B., Ball, C. D., Hamner, C. L., and Sell, H. M. Effect of 2,4-dichlorophenoxyacetic acid on the alpha- and beta-amylase activity in the stems and leaves of red kidney beans. Science **111**: 118. 1950.

8. Olsen, K. L. Effect of 2,4-D on the phosphatase enzyme in *Zea mays* and *Vigna sinensis* seedlings. Abstr. Western Section Meeting, Amer. Soc. Plant Physiol. 1950.
9. Sumner, J. B. and Somers, G. F. Chemistry and Methods of Enzymes, Second Edition. Academic Press, New York. 1947.
10. Sumner, J. B., Chou, T. C., and Bever, A. T. Phosphorylase of the Jack Bean: Its purification, estimation and properties. Arch. Biochem. **26**: 1–5. 1950.
11. Wort, D. J. The response of buckwheat to treatment with 2,4-dichlorophenoxyacetic acid. Amer. Jour. Bot. **36**: 673–676. 1949.
12. Wort, D. J. Effects of non-lethal concentrations of 2,4-D on buckwheat. Plant Physiol. **26**: 50–58. 1951.

OPTIMUM CHLORIDE CONCENTRATION FOR MALT DIASTASE ACTIVITY AS A FUNCTION OF TEMPERATURE

H. C. EYSTER

THE CHARLES F. KETTERING FOUNDATION, ANTIOCH COLLEGE,
YELLOW SPRINGS, OHIO

Received April 30, 1952

The chloride ion has long been known to accelerate and stabilize the dextrinizing activity of α-amylase derived from plant (**5**) and animal tissues (**9**). WARBURG and LÜTTGENS (**11**) observed recently that the photochemical production of oxygen by washed chloroplast fragments is similarly stimulated by the chloride ion. ARNON and WHATLEY (**1**) confirmed this discovery and presented evidence in support of their conclusion that the chloride ion activates the chloroplast fragments indirectly by protecting them against injury by light. GORHAM and CLENDENNING (**4**) reinvestigated the mechanism of chloride activation, rejected the light injury hypothesis, and concluded that the stimulating action of chloride on both chloroplasts and α-amylase has a common physical basis. Striking similarities between these two *in vitro* systems were indicated with respect to the concentration of chloride required to achieve maximum stimulation, relative effectiveness of other anions, and dependence of chloride activation upon pH (**4**).

The quantity of chloride which is required to effect maximum stimulation and protection of either α-amylase or chloroplasts over a wide temperature range has not been investigated previously. The thermolability of chloroplasts makes them quite unsuitable for such a study. On the other hand, α-amylase is less susceptible to thermal inactivation, a property which was utilized by OHLSSON (**8**) and KNEEN *et al.* (**6**) in separating α- and β-amylase. This paper reports experiments on the quantity of chloride which is required for maximum stimulation of malt diastase over a wide temperature range (20 to 60° C).

The standard reaction mixture was 50 ml. of 1% soluble starch, 40 ml. of buffer solution at pH 7, x ml. 0.5% commercial malt diastase solution, and 10-x ml. water. The total reaction volume was 100 ml. The starch solution was freshly prepared (Coleman and Bell, soluble starch, c.p.). MacIlvaine buffer (**7**) was used in maintaining a pH of 7.0. The diastase solution was freshly prepared (0.5% Merck's malt diastase, medicinal grade). After allowing it to stand one hour at room temperature, the enzyme solution was filtered through a heavy asbestos mat to provide a clear solution. Chloride introduced as a contaminant in the substrate and enzyme solutions was determined by the Volhard titration method (**10**). The observed chloride content was found to be too small to necessitate the introduction of a correction (< 0.5 mg. per 100 ml. of reaction mixture).

In each activity measurement, the reaction mixture without enzyme was mixed and adjusted to the reaction temperature. The enzyme solution was then added with stirring, and the time required for the disappearance of the iodine-starch color was determined by successive external tests with I_2-KI solution (**2, 3**). Exploratory determinations were conducted first in order to find the approximate salt requirement for a given temperature, and then

TABLE I

OPTIMUM NaCl CONCENTRATION FOR MALT DIASTASE ACTIVITY AT 20° C.

NaCl concentration	Time for digestion past last iodine-staining stage
mg./100 ml.	*min.*
30	70
30	70
40	64
40	64
50	68
50	68
60	72
60	71

a more precise study was made in the region of the indicated optimum salt concentration.

Table I presents the refined determination for the optimum NaCl concentration at 20° C. Three milliliters of 0.5% filtered diastase was used in this case. Table II reports the optimum salt concentration for malt diastase activity at 20, 25, 40, 50 and 60° C. For each 20-degree rise in temperature there was an approximately 10-fold increase in the chloride requirement for maximum diastase activity. The optimum chloride concentration for malt diastase at 20° C is slightly below 10^{-2} M, which is in agreement with

TABLE II

OPTIMUM NaCl CONCENTRATION FOR MALT DIASTASE ACTIVITY AT DIFFERENT TEMPERATURES.

Temperature	Optimum NaCl concentration
°C	*mg./100 ml.*
20	40 (0.0068 M)
25	60 (0.01 M)
40	400 (0.068 M)
50	1100 (0.19 M)
60	about 6000 (1.03 M)

earlier observations on α-amylases of various sources as well as on isolated chloroplasts.

The author is indebted to Dr. Kenneth A. Clendenning of these laboratories for suggestions in the preparation of the manuscript and to Lucy Birtle and Kenneth Webb for technical assistance.

LITERATURE CITED

1. Arnon, D. I. and Whatley, F. R. Is chloride a coenzyme of photosynthesis? Science **110**: 554–556. 1949.
2. Eyster, H. C. Auxin action. Science **97**: 358–359. 1943.
3. Eyster, H. C. Effect of auxins on the action of diastase *in vitro*. Plant Physiol. **21**: 68–74. 1946.
4. Gorham, P. R. and Clendenning, K. A. Anionic stimulation of the Hill reaction in isolated chloroplasts. Arch. Biochem. Biophys. **37**: 199–223. 1952.
5. Haehn, H. and Schweigart, H. Zur Kenntnis der Kartoffelamylase. Biochem. Z. **143**: 516–526. 1923.
6. Kneen, E., Sandstedt, R. M., and Hallenbeck, C. M. The differential stability of the malt amylases—separation of the α- β- compounds. Cereal Chem. **20**: 399–423. 1943.
7. Lange, N. A. Handbook of Chemistry. Third Edition, p. 907. Handbook Publishers Inc., Sandusky, Ohio. 1939.
8. Ohlsson, E. The two components of malt diastase. Z. Physiol. Chem. **189**: 17–63. 1930.
9. Sumner, J. B. and Somers, G. F. Chemistry and Methods of Enzymes. Second Edition, p. 106. Academic Press, Inc., New York. 1947.
10. Talbot, H. P. Qualitative Chemical Analysis. P. 130. The Macmillan Company, New York. 1931.
11. Warburg, O. and Lüttgens, W. [Photochemical reduction of quinone in green granules.] Biokhimiya **11**: 303–322. 1946.

AN AUTORADIOGRAPHIC TECHNIQUE FOR DETAILED STUDIES WITH PLANT TISSUE

W. M. DUGGER, JR. AND D. E. MORELAND

DEPARTMENT OF BOTANY, UNIVERSITY OF MARYLAND, COLLEGE PARK, MARYLAND AND COLLEGE OF FORESTRY, STATE UNIVERSITY OF NEW YORK, SYRACUSE, NEW YORK

Received May 5, 1952

Within the past few years, reports in the literature indicate the increasing use of detailed autoradiographic techniques in problems concerned with the location of areas of deposition or concentration of radioactive isotopes in animal tissue. The usefulness and value of these techniques has increased with the availability of special nuclear track emulsions and the development of suitable laboratory procedures. These autoradiographic techniques have not been fully utilized in studies of plants although ARNON *et al.* (**1**), COLWELL (**2**), HARRISON *et al.* (**8**), and GROSS and SNYDER (**6**) have used the gross or survey autoradiographic techniques. Several procedures have been used in conjunction with animal studies; and with minor modifications, these can be adapted to plant materials. A general review of these techniques as applied to animal studies has recently appeared in the literature (**7**). One of the techniques provides for a permanent mounting of the histological sections on the photographic emulsion throughout the exposure and processing periods, and it is this one that was used in the study. This method was first used with animal tissue by ENDICOTT and YAGODA (**4**) and EVANS (**5**). Microscopic examination with both high-dry and oil-immersion objectives is possible, and the deposited silver grains can be correlated with the tissues and cells containing the radioisotope.

Sections taken from Hydrangea leaves that had been exposed to an atmosphere containing radioactive carbon dioxide ($C^{14}O_2$) for four hours were used in this study. Details of the chamber which was used and the procedure which was followed have been previously reported (**3**). At the end of the period of photosynthesis, the leaves were separated from the plants and rinsed with dilute hydrochloric acid to remove adsorbed $C^{14}O_2$. Small strips were cut from the leaves and placed in an FAA fixing solution. The leaf tissue was then dehydrated and embedded in paraffin following usual procedures. Sections 15 microns in thickness were cut on a rotary microtome. Praffin ribbons of the desired length were expanded in warm water at 42° C. Following expansion, the ribbons were transferred to water at 18 to 20° C. The ribbons hardened at these lower temperatures and could be handled more easily during subsequent operations.

In the dark room, Eastman nuclear track plates of the NTB or NTB2 type were soaked in distilled water at 20° C for five minutes. The ribbons were removed from the water with a section lifter or a small hair brush and placed on the swollen emulsion. This manipulation was conducted under a Wratten safelight, series 2. Soaking the plates prior to placing the ribbons in contact with the emulsion ensures good adhesion between sections and

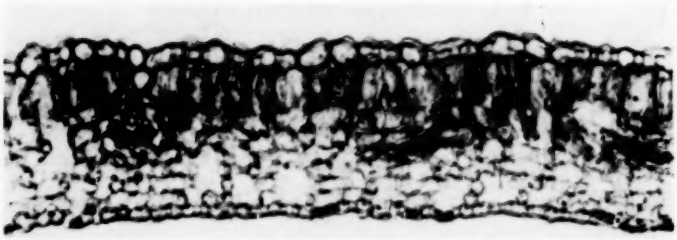

FIG. 1. Photomicrograph of a transverse section of a Hydrangea leaf (×170). Photographed through a green filter.

emulsion and eliminates the use of an adhesive. With the sections in place, the plates were positioned on top of small corks in light-tight boxes. Calcium chloride was placed in the bottom of the boxes as a desiccant, and the plates were stored in a refrigerator for the period of exposure.

At the end of the exposure period the paraffin was removed by immersing the plates in xylene for 10 minutes. The xylene was then allowed to evaporate completely from the plates. Plates were developed without agitation in Kodak D-19 developer for 20 minutes at a temperature of 20° C. The plates were then rinsed in water, fixed in Kodak F-5 for twice the clearing period, and washed in running tap water for one hour. Immediately following washing, and without permitting the plates to dry, the sections were stained in Harris' haematoxylin for 20 minutes. Destaining in acid water can be used to reduce the intensity of the stain wherever necessary. Selection of the appropriate time interval will eliminate this step. Sections were then blued in alkaline water to the desired degree. The plates were passed through a dehydration series and were counterstained for approximately one minute with either eosin, yellowish-acid, or erythrosin, bluish-acid. The final xylene-alcohol wash of the dehydration series had been saturated with the desired stain. The use and selection of a counterstain will depend on the requirements of the worker. The plates were transferred to xylene and mounted immediately in balsam which produced a slide with a stained tissue section mounted permanently on the emulsion.

Figure 1 is a photomicrograph of a transverse section of a Hydrangea leaf. The leaf was exposed to 1% $C^{14}O_2$ for four hours at a light intensity of 260 foot-candles. The section was mounted on the emulsion and proc-

FIG. 2. Composite photomicrograph and autoradiograph of the same section as in figure 1 (×170). Photographed through a red filter.

essed as outlined in the preceding section. Cellular detail can be observed in this focal plane. Shifting the focus to the underlying emulsion, as in figure 2, shows concentrations of silver grains corresponding to cellular fixation of the C^{14}. The stained cellular material of the leaf is easily distinguishable from the reduced silver grains of the autoradiograph. The leaf sections were 15 microns in thickness and were mounted on Eastman NTB2 plates having an emulsion thickness of 10 microns. The exposure interval was 26 days. The most dense concentration of deposited silver grains is correlated with cells of the palisade region. Untreated control sections showed only the normal background of grain formation. Green and red filters were used in preparing the photomicrographs to accentuate cellular details and the deposited silver grains.

The use of the isotope C^{14} as a source of radiation incorporated in plant material is ideal for autoradiographs. No special technique is required to prevent diffusion of C^{14} from the tissue during the preparations if it is in a fixed condition. The long half-life of C^{14} eliminates the necessity for rapid processing, and it is possible to use long exposure periods when the isotope is present only in minute quantities. In addition, the relative consistence in radiation activity permits accurate estimate of the time required to obtain an image of the desired density.

The authors wish to acknowledge the assistance of the Office of Naval Research and the Atomic Energy Commission in conjunction with this study.

LITERATURE CITED

1. Arnon, D. I., Stout, P. R., and Sipos, F. Radioactive phosphorus as an indicator of phosphorus absorption of tomato fruit at various stages of development. Amer. Jour. Bot. **27**: 791–798. 1940.
2. Colwell, R. V. The use of radioactive phosphorus in translocation studies. Amer. Jour. Bot. **29**: 798–806. 1942.
3. Dugger, W. M., Jr. Membrane permeability of CO_2. Research Rev. August, 1950.
4. Endicott, K. M. and Yagoda, H. Microscopic historadiographic technique for location and quantitative radioactive elements in tissues. Proc. Soc. Exp. Biol. Med. **64**: 170–172. 1947.
5. Evans, T. C. Radioautographs in which the tissue is mounted directly on the photographic plate. Proc. Soc. Exp. Biol. Med. **64**: 313–315. 1947.
6. Gross, A. V. and Snyder, J. C. Radioautographic with C_{14}. Science **105**: 240–241. 1947.
7. Gross, J., Bogoroch, R., Nadler, N. J., and Leblond, C. P. The theory and methods of the radioautographic localization of radio-elements in tissues. Amer. Jour. Roentgenol. and Rad. Therapy **65**: 420–458. 1951.
8. Harrison, B. F., Thomas, M. D., and Hill, G. R. Radioautographs showing the distribution of sulphur in wheat. Plant Physiol. **19**: 245–257. 1944.

NOTES

Western Section.—The Western Section of the A.S.P.P. met with the Pacific Division, A.A.A.S., and affiliated societies at Oregon State College, Corvallis, June 17–19, 1952. In addition to four sessions of submitted papers, two symposia were held jointly with the Botanical Society of America and the American Society for Horticultural Science: The Role of Cellular Components in Plant Metabolism, Sam G. Wildman, Chairman, and Morphological Approaches to Physiological Problems with Louis K. Mann as Chairman. Officers of the Section for 1952–53 are Chairman, Moyer D. Thomas, American Smelting and Refining Co.; Vice-Chairman, Harlan K. Pratt, University of California, Davis; and Secretary-Treasurer, D. J. Wort, University of British Columbia. The next meetings will be June 16–19, 1953, in Santa Barbara, California. Any members of the Society who may be able to attend should communicate with Prof. D. J. Wort, Department of Botany, University of British Columbia, Vancouver, B. C.

Alfred Ursprung.—Dr. Ursprung died April 21, 1952, in Freiburg, Germany, after an illness of about one year. Dr. Ursprung began his work for the doctorate in botany under Schimper in 1900 after preliminary studies in physics and mathematics at Basel and Strassburg. Early in his career, he competed successfully in writing a prize paper on "The physical properties of foliage leaves." Another important early publication was his work on "The mechanism of spore dispersal by Pteridophytensporangia." In the same year Dr. Ursprung assumed his post at Freiburg as director of the Botanical Institute which he served continuously for 47 years. He completed well over 100 papers of his own during this period and directed the studies of some 40 successful candidates for the doctorate in the field of plant physiology. Dr. Ursprung's best-known publications are on dispersal mechanisms of sporangia and anthers, radial growth in woody stems, sap ascent and osmotic phenomena connected therewith. In later years he studied the influence of light in relation to photosynthesis and the general problem of water balance in plants. No less important were his publications on terminology and methods as these are related to cell physiology. His work on water absorption has proved one of his most outstanding contributions. Dr. Ursprung was elected as a corresponding member of the American Society of Plant Physiologists in 1937.

Elizabeth S. Semmens.—The American Society of Plant Physiologists records with sorrow the passing of one of its members, Elizabeth S. Semmens, West Grove, Sandgate, Kent, England, in June 1952. Dr. Semmens had been a member of the Society since 1946 and published work on the hydrolysis of organic products by polarized radiation.

Plant Physiology.—B. S. Meyer and D. B. Anderson. Second Edition. D. Van Nostrand Company, Inc., 250 Fourth Avenue, New York 3, New York. 784 pages, 1952. $8.50.

Members of the profession engaged in teaching plant physiology will welcome the revised and enlarged second edition of this popular textbook which had 13 reprintings in the first edition. The general organization of the material is essentially unchanged. However, those portions dealing with basic physicochemical principles and those dealing with water relations of plants have been shortened. Whereas an entire chapter was devoted to a discussion of permeability in the first edition, this topic is treated in only a few pages in the present volume. The chapters dealing with metabolism have been revised completely to include advances made during recent years in the knowledge of metabolic processes in plants. The chapters on growth have been reorganized to include a discussion of vegetative growth and the environmental factors affecting it, and a discussion of reproductive growth and the environmental factors influencing such growth. The bibliographies at the end of the chapters include references to the literature through 1951. An index by author and another by subject are included in the book.

Paper Chromatography (A Laboratory Manual).—R. J. Block, R. Le-Strange, and G. Zweig. Academic Press, Inc., 125 East 23rd Street, New York 10, New York. 195 pages, 1952. $4.50.

This monograph is intended to contain sufficient detail to be used primarily as a practical manual of proven procedures requiring relatively simple equipment; however it seems doubtful that the average investigator would not wish to refer to original literature also. Following a brief introduction are chapters dealing with theory; general methods; quantitative methods—illustrated with amino acids; amino acids, amines, and proteins; carbohydrates; aliphatic acids and steroids; purines, pyrimidines, and related substances; phenols, aromatic acids, and porphyrins; miscellaneous organic substances; antibiotics and vitamins; and inorganic separations. There is a bibliography containing nearly 400 references of which a great majority are of the period 1948 through 1951, but not all are referred to in the text. An author index and a subject index are included.

Annual Review of Biochemistry, Volume XXI.—Edited by J. Murray Luck. Annual Reviews, Inc., Stanford, California. 781 pages, 1952. $6.00.

This volume consists of 23 articles of which the following will be of special interest to plant scientists: Proteolytic Enzymes by A. K. Balls and E. F. Jansen; Nonoxidative, Nonproteolytic Enzymes by K. I. Altman and A. L. Dounce; Chemistry of the Carbohydrates by R. Montgomery and F. Smith; Chemistry of Lipids by H. J. Deuel, Jr. and R. Alfin-Slater; The Chemistry and Metabolism of the Steroids by L. T. Samuels and H. Reich; The Chemistry of Amino Acids and Proteins by H. B. Bull; Nucleic Acids, Purines, and Pyrimidines by D. O. Jordan; Fat Metabolism by A. C.

Frazer; Interrelationships of Lipid and Carbohydrate Metabolism by K. Bloch; Metabolism of Amino Acids and Proteins by H. Tarver; Fat-Soluble Vitamins by A. R. Kemmerer; The Biochemistry of Antibiotics by T. S. Work; Carotenoids by G. Mackinney; The Alkaloids by L. J. Sargent and L. F. Small; Chromatography by S. Moore and W. H. Stein; Carbohydrate Metabolism by S. Ochoa and J. R. Stern; Biological Oxidations by B. Change and L. Smith. Author and subject indices are included in the volume.

Advances in Agronomy, Volume IV.—A. G. Norman, Editor. Academic Press, Inc., 125 East 23rd Street, New York 10, New York. 416 pages, 1952. $8.50.

The articles in the fourth volume of this series present various types of reviews: Grassland Agronomy in Australia by H. C. Trumble; Type of Soil Colloid and the Mineral Nutrition of Plants by A. Mehlich and N. T. Coleman; The Physiological Basis of Variation in Yield by D. J. Watson; Copper in Nutrition by F. A. Gilbert; Ecological and Physiological Factors in Compounding Forage Seed Mixtures by R. E. Blaser, W. H. Skrdla, and T. H. Taylor; Soil Manganese in Relation to Plant Growth by E. G. Mulder and F. C. Gerretsen; Atomic Energy and the Plant Sciences by N. E. Tolbert and P. B. Pearson; Vegetation Control on Industrial Lands by K. C. Barrons; Soil and the Growth of Forests by T. S. Coile. Author and subject indices are included.

PREPARATION OF MANUSCRIPTS FOR PUBLICATION IN PLANT PHYSIOLOGY

The following suggestions are made to authors writing papers for this journal in order to minimize revisions and editorial corrections. Though many points may appear arbitrary or trivial, attention to details is necessary to secure uniformity of format and style. The membership dues and subscription fees go almost entirely to publication costs, as all of our editors give their services without financial recompense. Hence the cooperation of authors in preparation of manuscripts minimizes expense and permits greater liberality as to length and number of papers published. Authors will find the following concise publications useful in preparation of manuscript copy: A. J. Riker, The Preparation of Manuscripts for Phytopathology. PHYTOPATHOLOGY XXXVI, pages 953–977, 1946; and S. F. Trelease, The Scientific Paper: How to Prepare It. Second Edition. Williams and Wilkins Company, Baltimore, Maryland, 1951.

1. **Length.** The allowable length of papers is 20 printed pages, with tables, figures, formulae, and equations not to exceed 25% of the total. Authors will be billed at cost for overrun and excess of figures and tables.

2. **Format.** In the preparation of typescripts, authors are requested to examine a printed copy of the journal of PLANT PHYSIOLOGY to note the position of headings, general organization, punctuation, abbreviations, and the methods of citing literature particularly, in order to bring the script into conformity with the general style of the journal. Leave a three inch margin at the top of the first page, liberal side margins, and double spacing of lines to permit proofmarking and required editorial changes.

3. **Headings.** Leave all headings unscored and scientific names without underlining, as the editors will proofmark these items in accordance with the practice of the journal. Generic names used alone are usually capitalized but not italicized. In general, do not underscore any lines.

4. **Footnotes.** Avoid footnotes as far as possible by placing such items under acknowledgments or at end of summary. When footnotes are used, they should be numbered with Arabic numerals.

5. **Figures.** Copy for figures (photographs and drawings) should not exceed $8\frac{1}{2} \times 11$ inches. Retain original drawings as a safeguard against loss. If the figures submitted are larger than $8\frac{1}{2} \times 11$, they will be photographed at this office and the author will be billed for the same. Author's name and figure number must be written on the back of copy for each figure. Do *not* put legends into or on the figure, either front or back. Type all legends *double* spaced, properly numbered to correspond to the figures, on a *separate sheet* of your manuscript. Graphs and drawings should be inked with fairly heavy lines and with lettering of approximately uniform size to insure clarity of printed figures after reduction. Photographic prints should be submitted on gloss-finish paper. Limit copy to desired visual details, trim off irrelevant foreground and minimize blank space, as charges for cuts are based on area. Unless otherwise directed, the editor will proofmark copy to determine actual size of printed figures. Old cuts will be sent to authors after printing for cost of postage if printer is so directed when galley proof is submitted.

6. **Tables.** Place each table on a separate page; do not crowd too much material into a single table. Note size of printed page (width and length, $4\frac{1}{2} \times 7\frac{1}{2}$ inches) and gauge tables accordingly, leaving ample space in margins and in the interior for editorial marks. If at all possible, arrange tabular data horizontally on the page in order to obviate tilting of printed table. Use horizontal lines (rules) at top and bottom, but omit or employ such rules sparingly in interior of tables. Use vertical rules between columns. Rules and spaces (leads) are usually used in the final printed tables to separate sets of data. For footnotes to tables use the asterisk, double asterisk, dagger, double dagger, and section in this sequence as needed. Tabular material should be used sparingly because of its high cost.

7. **Acknowledgments.** Place acknowledgments at the end of the paper just below summary.

8. **Address.** The name of your institution and its address should be listed just below the names of the authors.

9. **Literature Cited.** Use separate pages for the literature cited and type *double spaced*. Give complete citations, author, title, journal name, volume number, inclusive pages, and year. (See any literature list in a recent issue of the journal for examples). Punctuate according to the examples, and leave no citations incomplete. Write out names of states in full. Note proper abbreviations of journals. An error frequently noted in manuscripts with long bibliographies is failure to mention in the text all citations listed. Authors are requested to check all citations in the bibliography by careful proofreading of manuscripts prior to submission.

10. **Formulae.** Use mathematical formulae only when necessary, and be conservative in the use of space.

11. **Galley Proof.** Correct all errors clearly and return galley promptly to editor. Authors will be billed at cost for alterations in the galley proofs, other than corrections of printer's errors, involving excessive changes from manuscript copy.

12. **Mailing.** Send original copy of manuscript to editor, W. F. Loehwing, University of Iowa, Iowa City, Iowa. Retain a carbon copy to insure against loss, and to consult in case of need. Prompt return of revised manuscript copy and galley proof to editor greatly expedites publication. Write across top of title page of manuscript directions for mailing of proof, thus: Mail proof to (author's name and address). If authors wish to have original manuscripts or copy for illustration returned subsequent to publication, the editor should be so informed at the time proof is returned.

13. **Reprint Order.** These should be sent to the editor with corrected galley proof. Late orders for reprints entail extra work and expense by printer for which charges must be made to author. Type is destroyed immediately after printing and hence it becomes impossible to supply reprints after publication of papers. Send Reprint Order with *corrected galley* even if formal requisition or purchase order for institutional agencies follow later.

14. **Abstracts.** Authors are requested to submit typed abstract on form provided when corrected galley is returned to editor who will insert proper volume and paging for use by Biological Abstracts.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

Organized in 1924 for the advancement of Plant Physiology as a pure and applied field of botanical science

---

OFFICERS FOR 1952–1953

President: Dr. Daniel I. Arnon,
University of California, Berkeley, California

Vice-President: Dr. Folke Skoog,
University of Wisconsin, Madison, Wisconsin

Secretary: Dr. Alden S. Crafts,
University of California, Davis, California

Executive Secretary-Treasurer: Dr. J. Fisher Stanfield,
Miami University, Oxford, Ohio

Any one actively interested in Plant Physiology is eligible for membership in the Society. Candidates for membership must be recommended by two members, and approved by executive committee, or by the vote of the Society at some annual or special meeting.

The payment of the annual dues to the Society entitles the members to receive the official journal, Plant Physiology. They will receive in addition the regular bulletins and announcements sent out periodically by the secretary. Members are invited to take a personal interest in the growth and development of the Society.

Manuscripts for Plant Physiology should be addressed to Prof. Walter P. Loomis, Department of Botany, State University of Iowa, Iowa City, Iowa. Drawings and photographs should not exceed 8.5 × 11 inches in size, should be numbered correctly on the back, and separate typewritten legends included with the text of the manuscript. Tables and cuts should be used sparingly, and the text presented in concise form. Instructions to authors for preparation of manuscripts are given in the January number, Volume 28, 1953, of Plant Physiology, 149–150.

(CONTENTS—*Continued*)

The Relation of the Amino Acid Composition to the Development of Oats. THOMAS A. MCCOY, THOMAS H. SUBLETT and VERGIL W. DOBBS, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma 77

Electrode Reactions of Isolated Chloroplast Fragments. H. S. A. GILMOUR, RUFUS LUMRY and JOHN D. SPIKES, Department of Chemistry and Division of Biology, University of Utah, Salt Lake City, Utah 89

Effect of Inorganic Ions and Their Conductances on Geotropic Curvature of the Avena Coleoptile. A. R. SCHRANK, Department of Zoology, University of Texas, Austin, Texas 99

Filters for the Isolation of Narrow Regions in the Visible and Near-Visible Spectrum. ROBERT B. WITHROW and LEONARD PRICE, Division of Radiation and Organisms, Astrophysical Observatory, Smithsonian Institution, Washington, D. C. 105

Phosphogluconic Dehydrogenase in Higher Plants. ROBERT C. BARNETT, HELEN A. STAFFORD, ERIC E. CONN and BIRGIT VENNESLAND, Department of Biochemistry and Department of Botany, University of Chicago, Chicago, Illinois 115

Brief Papers:

Frenching in Ragweed (Ambrosia artemisiifolia L.). LINUS H. JONES, Department of Botany, University of Massachusetts Agricultural Experiment Station, Amherst, Massachusetts 123

Effect of Stable Boron Isotopes on Growth of Tobacco in Solution Cultures. J. E. MCMURTREY, JR. and H. B. ENGLE, Bureau of Plant Industry, Beltsville, Maryland 127

Low Temperature Inductions of Flowering in a Nicotiana rustica × N. tabacum Hybrid. ROBERT A. STEINBERG, Bureau of Plant Industry, Beltsville, Maryland 131

The Effect of 2,4-Dichlorophenoxyacetic Acid on Phosphorylase, Phosphatase, Amylase, Catalase, and Peroxidase Activity in Wheat. D. J. WORT and L. M. COWIE, Department of Botany, University of British Columbia, Vancouver, British Columbia 135

Optimum Chloride Concentration for Malt Diastase Activity as a Function of Temperature. H. C. EYSTER, The Charles F. Kettering Foundation, Antioch College, Yellow Springs, Ohio 140

An Autoradiographic Technique for Detailed Studies with Plant Tissue. W. M. DUGGER, JR. and D. E. MORELAND, Department of Botany, University of Maryland, College Park, Maryland, and College of Forestry, State University of New York, Syracuse, New York 143

Notes 146